CELL-CYCLE ENGINEERING AND GFP-ONLINE MONITORING OF HETEROLOGOUS PROTEIN PRODUCTION IN HIGH DENSITY PLANT SUSPENSION CULTURES

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ABSTRACT

Plant suspension culture is an effective system for producing high-value recombinant protein products. In this study, two synergistic approaches have been taken to enhance the recombinant protein productivity of bioreactor-based plant cell culture. First, the high-density transgenic *N. tabacum* suspension cells were continuously cultivated to enhance recombinant protein production in a perfusion stirred tank reactor (STPR). Systematically, 83% increase in recombinant protein productivity was achieved under high-density plant suspension culture that maintained in a perfusion stirred tank reactor. Furthermore, we proposed a GFP-fluorescence sensing model. It offers an effective means for online compensation of IFE (inner filter effect) to enable quantitative interpretation of the culture fluorescence signals for accurate reporting of GFP or GFP-fusion protein expression. Based on Extended Kalman Filter, a dynamic state model of plant cell culture and the GFP-fluorescence sensing model, a state observer had been successfully developed for monitoring various transgenic tobacco suspension culture states (GFP/GFP-fusion protein expression, culture growth, glucose consumption) in bioreactor.

Secondly, controlled-proliferation by genetically arresting cell cycle progression has been shown to increase the production of heterologous recombinant proteins in mammalian cell cultures. In this study, we examined the applicability of such approach in plant cell cultures. Flow cytometry analysis of the ICK-1 expressing cells revealed a high degree of cell cycle arrest at G1/S, demonstrating the effectiveness of inducible ICK-1 expression in controlled proliferation of culture plant cells. However, in contrast to the mammalian systems, the recombinant reporter protein production was not improved
the mammalian systems, the recombinant reporter protein production was not improved as a result of ICK-1 expression. About 3-fold decrease in specific productivity of GFP was observed upon expression of ICK1. A lower cellular metabolic activity (slower glucose consumption rate, lower culture viability, reduced total protein synthesis) was observed in those ICK1-expressing *N. tabacum* suspension culture. We also noted a reduction in GFP transcripts (determined by quantitative RT-PCR) after ICK1 expression. These data suggest the reduced GFP productivity from ICK-1 expression may result from both decreased metabolic activity and cell-cycle dependency of the CaMV 35S promoter.
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CHAPTER 1. Literature Review

1.1 Culturing high-density plant suspension culture

1.1.1 Plant cell suspension culture

Plant cell suspension culture has been suggested as a potential system to produce small-to-medium quantities of high-value medical proteins (Kwon et al. 2003), enzymes (Villegas and Brodelius 1990), recombinant antibodies, as well as secondary metabolites as pharmaceuticals or food additives (Yamamoto et al. 2001). Production of high-value pharmaceutical recombinant proteins by fermentation of plant suspension culture in bioreactor can circumvent problems such as environmental variation, insect disease, high expense to recover products and heterogeneous properties of crops associated with the traditional agricultures. The growth rate of the cell suspension culture is generally higher than that of whole plants and suspension cell cultures are amenable to scale-up. Additionally, it has been suggested that plant cells cultured in liquid media are less susceptible to post-transcriptional gene silencing (PTGS) that is usually triggered in limited number of cells and spread systemically through plasmodesmata (de Wilde et al. 2000). On the down side, limitations of plant cell suspension culture compared to microbial or mammalian culture include high viscosity due to the high biomass concentration of plant suspension cultures, leading to mixing problems (Tanaka 1981) and the long doubling time of plant cells, which requires long culture period and stricter requirements in culture sterility. Overall, plant cell suspension culture is still considered an essential and important host for production of a large number of pharmaceutical proteins or plant metabolites due to its advanced post-translational processing machinery.
over microbial culture and less cost than mammalian culture (Sijmons et al. 1990; Simmons et al. 1991; Su et al. 1996).

1.1.2 Recombinant protein production by culturing plant cells in perfusion bioreactor

Perfusion bioreactor has been widely practiced in mammalian cell cultivations to enhance cell viability by replenishing nutrients. In recent years, perfusion bioreactor has begun to be applied in plant suspension cell cultivation, to produce plant secondary metabolites such as rosmarinic acid (Su et al. 1993) and berberine (Kim et al. 1991), secreted proteins and enzymes (Li et al. 2003; Su and Arias 2003; Su et al. 1996) and also high-value recombinant protein such as hGM-CSF (Lee et al. 2004). One main advantage of perfusion plant cell culture is to operate the culture at high cell density over an extended period to achieve a higher productivity. The continuous perfusion can substantially prevent the degradation of secreted protein caused by proteolysis in plant cell culture (Lee et al. 2004). The secreted enzyme and recombinant protein accumulated and removed in the spent medium overflow can be coupled to downstream purification and make product recovery more cost effective.

1.2 Green fluorescent protein (GFP) as reporter for monitoring transgenic plant cell cultures

1.2.1 Green fluorescent protein (GFP)

A protein, existing in a large number of bioluminescent coelenterates, was isolated from jellyfish *A. victoria* and found to give green fluorescence (Shimomura et al. 1962). The gene encoding this protein was cloned and sequenced (Prasher et al. 1992). This protein,
known as the green fluorescent protein, had been widely used in prokaryotic and eukaryotic organisms to label protein for cellular localization studies by genetic tandem fusion (Cubitt et al. 1995). In addition to its applications in basic life sciences, GFP technology is also applicable in high throughput drug screening, evaluation of viral vectors for human gene therapy, and biological and environmental control of microbes (Chalfie and Kain 1998). The green fluorescent protein will be introduced below based on its fluorescent and biochemical properties, structures, and its applications in monitoring various bioprocesses.

1.2.1.1 Fluorescent and biochemical properties of GFP

Biologically, in coelenterates, the green fluorescent protein converts the blue bioluminescence generated by aequorin, a protein regulated by calcium ion level, into green fluorescence. Such a spectrum shift happens by both radiative energy transfer (excited light generate by donor molecules was absorbed by acceptor molecules and reemitted by the acceptor) and radiationless energy transfer (non-photon emission between donor and acceptor) (Ward and Cormier 1979). The excitation peak of wild-type green fluorescence protein is at 395 nm and a minor peak at 475nm. The green fluorescence is emitted at 508 nm (Kahana and Silver 1996; Ward and Cormier 1979). The fluorescence of GFP does not require any other cofactor or substrate. The self-contained chromophore forms from the protein peptide backbone Ser-Tyr-Gly sequence (Chalfie and Kain 1998). The chromophore structure was proposed to resemble to a imidazolone ring (Shimomura 1979) and its side chains were confirmed by (Cody et al. 1993).
The high stability of GFP is also one of its advantages as a reporter protein: GFP was found to be highly resistant to heat. The T_m (temperature at which the endogenous fluorescence is lost) for all of Aequorea GFP, Renilla GFP and Phialidium GFP are ranging from 76°C to 69°C (Bokman and Ward 1981; Levine and Ward 1982). GFP remains its full fluorescence between pH 7.0 to pH 10. The fluorescence of Aequorea GFP (Chalfie and Kain 1998) and its recombinant isoforms (Crameri et al. 1996) is bleached when pH is lower than 4.7. The Aequorea GFP undergoes excitation shift when pH is higher than 12.6 (Gonzalez et al. 1997). In contrast, Renilla GFP has extremely high loss of fluorescence under alkaline conditions (Ward 1981). Although heat-denatured GFP does not renature effectively, fully denatured Renilla and Aequorea GFP by acid (pH 1.0), base (pH 13), 8M urea and 6M guanidine-HCl was demonstrated to recover most of its full fluorescence (Ward and Bokman 1982). Additionally, the Aequorea GFP fluorescence is able to tolerate as high as 1mg/ml protease (trypsin, chymotrypsin, papain, subtilisin, thermolysin) concentration (Bokman and Ward 1981) and a large number of anionic, cationic and nonionic detergents (Gonzalez et al. 1997) without impairments in fluorescence intensity. Such a stability of GFP is believed as a consequence of its unique three-dimensional structure (Chalfie and Kain 1998; Ormo et al. 1996; Yang et al. 1996).

1.2.1.2 Structures of GFP

The first cloned GFP cDNA comprised of 238 amino acids with monomer molecular weights of approximately 27 kDa. The Aequorea GFP is a compact, globular molecule and exists in form of monomers in aqueous solutions (Prendergast and Mann
1978). All other GFP are identified as stable, non-dissociable dimers unless denatured (Chalfie and Kain 1998). The crystal structure of the wild-type GFP was reported as a cylinder with regular β-barrels surrounded by 11 strands. The internal Ser-Tyr-Gly locating at 65-67 residues can form 4-(p-hydroxybenzylindene)-imidazolidin-5-one structure by post-translation modification (Chalfie and Kain 1998; Cody et al. 1993). Arginine at residues 96 forms a hydrogen bond with carbonyl oxygen of Ser\textsuperscript{65} and activates carbonyl carbon for nucleophilic attack by the amide nitrogen of Gly\textsuperscript{67} (Branchini et al. 1997). Followed by dehytrogenation of the α-β bond of residue 66, the aromatic ring on Tyr\textsuperscript{66} was conjugated with imidazoline, which grants the functional activity to the fluorophore. The fluorophore resides inside the cylinder as a part of a single α helix. This motif was known as β-can. On each end of the cylinder, a small α helix was formed (Yang et al. 1996). This tightly-sealed β-barrel protects fluorophore from oxidation photo-quenching, heat, denaturants and contributes high stability to GFP.

1.2.1.3 Application of GFP in plants and plant cell suspension cultures

During the past decades, green fluorescent protein was successfully expressed in a series of prokaryotic organisms, such as *Escherichia coli* (Chalfie et al. 1994), *Bacillus subtilis* (Arigoni et al. 1995), *Mycobacterium marinum* (Valdivia and Falkow 1996) as well as eukaryotic organisms: the wild type GFP and its variant have been expressed in many mammalian cells including BHK (Olson et al. 1995), CHO (Cole et al. 1996), HeLa (Kaether and Gerdes 1995) and GH3 (Ogawa et al. 1995) cells. A high level expression of wild-type GFP was detected in tobacco plants by using cytoplasmic RNA viruses potato virus X and tobacco mosaic virus (Baulcombe et al. 1995; Heinlein et al. 1995).
Although the wild-type GFP has been reported to be expressed in plant protoplasts of tobacco (Reichel et al. 1996), maize (Hu and Cheng 1995) and *Citrus sinensis* (Niedz et al. 1995), a much higher expression of mgfp5 (removal of cryptic intron) was demonstrated successful in a broader host system that involved in the aberrant GFP intron recognition (Luehrs en et al. 1994). In transgenic plant cells, GFP is prevalent in the cytoplasm, but accumulates in the nucleoplasm (Haseloff et al. 1997). However, by fusing to various targeting peptides to GFP, GFP was found to be localized in mitochondria (mitochondrial targeting sequencing from yeast cytochrome c oxidase IV protein), the lumen of the ER (HDEL), secretory pathway (carboxypeptidase Y and Arabidopsis basic chitinase). This enables GFP to be a marker for several subcellular organelles (Haseloff et al. 1997; Kohler et al. 1997).

In addition to the advantageous application of GFP in whole plants, it has also been proven a useful reporter in transgenic plant suspension cells. The mgfp5-ER was demonstrated to be effectively used for monitoring transgenic tobacco cell growth and protein production in bioreactor (Liu et al. 2001). By splicing an *Arabidopsis* basic chitinase signal peptide to GFP, (Su et al. 2004) used secretory GFP to quantify the secreted recombinant protein production in batch cultivation.

1.2.2 Online GFP fluorescence monitoring of transgenic tobacco suspension cultures

1.2.2.1 Inner filter effect and online fluorescence sensing model

Although the GFP fluorescence was demonstrated as an effective reporter for monitoring various bioprocesses, the biochemical fluorescence/bioluminescence is practically a composite signal significantly, and often fatally affected by a number of
factors (Srinivas and Mutharasan 1987). Absorption and scattering of both excitation and emission light by non-fluorescent compounds and fluorescent components inevitably impair the intrinsic accuracy of using fluorescence/luminescence. This phenomenon is known as the inner filter effect (IFE). The inner filter effect (IFE) is encountered in fluorophore (NADH) solutions, intrinsic fluorephore of *E.coli* suspension culture and yeast culture (Konstantinov and Dhurjati 1993; Liden and Niklasson 1993; Srinivas and Mutharasan 1987). It is especially problematical in transgenic plant cell suspension cultures, due to the aggregated nature of the cells and the high biomass concentration in these culture systems.

Principally based on the Beer-Lambert law, a number of approaches for compensating IFE were reported in real-time fluorescence monitoring bacterial cultures in bioreactor (Konstantinov et al. 1993; Li and Humphrey 1992; Lutz and Luis 1983; Srinivas and Mutharasan 1987; Wang and Simmons 1991). Srinivas and Mutharasan (1987) assumed both of the excitation light and the emission light are collimated beam perpendicular to the fluorescence probe tip. The fluorescent and non-fluorescent components are not interacting and their influence on IFE are additive. Wang and Simmons (1991) reported a modified model that takes into account the geometric fraction due to uniformly distribution of emission light. A geometric fraction of “partially distributed” emission light was also reported by (Liden and Niklasson 1993). In addition, (Modlin and Milanovich 1991) proposed a model for a cleaved optic fiber with nonparallel excitation light leaving the light rod fiber. Based on the model reported by Wang and Simmons (1991) which was modified from that of Srinivas and Mutharasan (1987), in this study we proposed an improved GFP fluorescence sensing model (for the
backscattering probe configuration) that accounts for the IFE by incorporating a modified geometric factor.

1.2.2.2 State Estimation using Extended Kalman Filter (EKF)

Although a quite number of kinetics and stoichiometric models of the plant suspension culture have been reported (Bailey and Nicholson 1989; Bailey et al. 1985; Curtis et al. 1991; Gunst et al. 1990; Pareilleux and Chaubet 1980; Van Gulik et al. 1992), it was found to be problematic to use mathematical models alone for predicting unknown state parameters, especially for intracellular contents (Ramirez 1994). During bio-reaction process, the inevitability of initial state error and system error can lead to model deviation from the true state values. To achieve superior performance to keep track of key culture states, an easily available online measurement model and a dynamic process model is coupled together by Extended Kalman filter algorithms, which not only reduces the measurement noises, it also enables estimation of states that are not readily measurable, and partly compensates for uncertain system dynamics (Stephanopoulos and Park 1991), and therefore expands the utility of the system models. Such a sensing approach can simultaneously detect various state parameters without implementing additional sensing instruments. EKF was employed for on-line estimation of microalgal photobioreactor culture states by conjugated with online-measure of local photosynthetic photon flux fluence rate (Su et al. 2003) or dissolved oxygen concentration (Li et al. 2003). In continuous yeast culture, EKF was used to estimate biomass concentration, maximum specific growth rate, saturation constant and substrate yield coefficient (Nahlik and Burianec 1988). Carbohydrate measurement linked to EKF was also used to estimate
the biomass concentration in plant cell cultures (Albiol et al. 1993). (Zhang and Su 2002) employed online oxygen uptake rate measurement to track of intracellular phosphate content in *Anchusa officinalis* culture. In this study, the online culture fluorescence generated by intracellular GFP or GFP fusion protein was modeled by effectively compensating for the inner filter effect in real time. This GFP fluorescence model was combined with unstructured batch kinetics and EKF to estimate cell growth, glucose consumption and GFP or GFP fusion protein production.

### 1.3 Plant cell cycle

Cell cycle is known as a process that precisely controls the cell division and proliferation, which ensures the cellular genetic information is replicated with adequate fidelity and cells divide under precise timing. The eukaryotic cell cycle consists of four phases: DNA replication phase (S phase) and cell division phase (M phase) and two gap phases (G1 phase and G2 phase) that separate S phase and M phase (Buchanan et al. 2000). Although the cell cycle core mechanism of eukaryotic cells is conserved and similar in plant cells and animal cells, the plants cell cycle possesses a series of specific distinctive features: the replication of three genomes (nuclear, mitochondrial, and plastid), the separation of the chromosomes by cell plate during mitosis, and specific cell cycle regulators (Buchanan et al. 2000). The environmental influence on plant cell cycle (Dewitte and Murray 2003) and unique plant cell cycle regulator sequences (Meyerowitz 2002) also led to independent view about plant cell cycle.
1.3.1 Key plant cell cycle regulators

1.3.1.1 Cyclin dependent kinases (CDKs) and Cyclins

The cyclin-dependent kinases (CDKs) are specific serine/threonine kinases that control progression through the cell cycle in all eukaryotes. Their activity is regulated both by association with cyclin regulatory subunits, by specific phosphorylation and dephosphorylation events (Huntley and Murray 1999). Currently, the multi-classes of cyclin dependent kinases (CDKs) are divided into four categories. CDK-a is widely identified in most plants. CDK-a carries the conserved PSTAIRE domain on its N-terminus which is responsible for cyclin binding (Mironov et al. 1999). The amino acids upstream CDK-a’s PSTAIRE domain involves in the ATP binding (Buchanan et al. 2000). The transcript level of CDK-a basically maintains constant throughout plant cell cycle (Fobert et al. 1996; Segers et al. 1996) and over-expression of CDK-a does not trigger special phenotype (Hemerly et al. 1995). The plant unique CDK-b consists of two sub-classes: CDK-b1 (containing PPTALRE domain) and CDK-b2 (containing PPTTLRE domain) (Fobert et al. 1996; Magyar et al. 1997). The CDK-c shares PITAIRE domain with the cholinesterase-related cell-division controller (CHED) and it does not interact with A, B, or D cyclins (Dewitte and Murray 2003). CDK-e was identified in both Arabidopsis and alfalfa based on the SPTAIRE motif. The function of CDK-c and CDK-e in plant cell cycle remains unclear so far.

Cyclins form a complex with CDKs and function as a regulatory subunit of CDKs, which confer the kinase activity to CDKs and specify the substrate specificity and subcellular localization of CDKs. Plant cyclins are classified into two main groups: mitotic cyclins (CycA and CycB) and G1 cyclins (CycD and CycE) (Buchanan et al.
2000; den Boer and Murray 2000; Renaudin et al. 1996). CycA and CycB cyclins both carry two conserved domains that are responsible for CDKs binding and mitotic protease degradation respectively. CycD cyclin is induced at specific cell cycle point by presence of mitogens (Huntley and Murray 1999) and expressed on a constant level in actively dividing cells (Sorrell et al. 1999). CycD cyclin was demonstrated to cross-react with CDKa in both tobacco BY-2 cells (Nakagami et al. 1999) and *Arabidopsis thaliana* cells. The Cyclin-CDK complexes are also regulated by a series of specialized kinases and phosphatases.

### 1.3.1.2 Kinases, phosphatases and inhibitors of CDK-cyclin complex

The CDK-cyclin complex is a highly regulated serine-threonine kinase. The CDK-cyclin complex is modulated by a series of kinases, phosphatases and specific inhibitors. The kinase activity of CDK-cyclin complex highly depends on its spatial configuration. A configurational domain ‘T-loop’, residing in between CDKs’ residues 146-166, regulates its substrate binding and phosphorylation, which can be “switched on” after the Thr-160 of CDK is phosphorylated. Such a specific fully-activating phosphorylation on CDK-cyclin is controlled by a class of proteins, CDK-activating kinase (CAK) (Fowler et al. 1998). On the other hand, the activity of CDK-cyclin can be inhibited through phosphorylating the Thr-14 and Tyr-15 by homologue of Wee1-type kinases (Sun et al. 1999). This inhibitory reaction is usually to allow the cells to arrest cell cycle and repair the damage during G2-M phase transition (Buchanan et al. 2000). Whereas, the inhibitory Thr-14/Tyr-15 phosphorylated CDK-cyclin can be re-activated by cdc25 phosphatase (Marcel and Murray 2001; Millar and Russell 1992). Furthermore, the plant CDK inhibitor (CKI), first identified in *Arabidopsis thaliana*, can inhibit the
active Thr-160 phosphorylated CDK-cyclin complex to cease cell cycle reversibly (Wang et al. 2000). In addition to the above mentioned kinases, phosphatases and inhibitors of CDK-cyclin complex, the CDK subunit proteins (Zhou et al. 2002b), retinoblastoma protein (Dewitte and Murray 2003) and E2F (Rossignol et al. 2002) were all demonstrated to interact with CDKs-cyclin, therefore function in cell cycle process.

1.3.2 Plant cell cycle progression

In both mammalian and plants, the target of the G1 CDK/cyclin complexes is the pRb/E2F pathway (Vincenzo and Serena 2002). During G1 phase, the transcription of D-type cyclins is activated by environmental and intrinsic signal, which leads to the formation of active CDKa-CycD complexes (Dewitte and Murray 2003; Marcel and Murray 2001). The CDKa-CycD phosphorylates the retinoblastoma protein (pRB) in late G1 phase and the phosphorylated pRB is inactivated and loses its binding to E2F. Therefore, E2F is released from the repressive pRB-E2F complexes and able to activate transcription of E2F-responsive genes involved in S phase and the cell enters S phase (Dewitte and Murray 2003; Huntley and Murray 1999; Marcel and Murray 2001; Vincenzo and Serena 2002). S phase progression is believed to be controlled by cyclin A kinases (Roudier et al. 2000). At G2/M transition point, the homologue of phosphatase cdc25 regulates the entry of cell cycle into M phase. CDK-CycB complexes are initially inactive due to inhibitory phosphorylation carried out by the wee1 kinase (Marcel and Murray 2001; Sun et al. 1999), but phosphatase cdc25 removes those inhibitory phosphate residues, after which cells enter M phase (Buchanan et al. 2000). Cdc25 phosphatase is found to regulate the timing of entry into mitosis in many organisms.
1.4 Improved protein production by arresting cell proliferation

1.4.1 Cell-cycle arrest for optimal recombinant protein production

The arrested cell-cycle led to cell division and cell growth block. The regulation of cell cycle allows the design of a novel two-stage production process: a proliferation phase leading to the desired cell density, followed by an extended production phase during which the cells remain growth-arrested and increase cell-specific production of the heterologous protein (Mazur et al. 1998). Such an increase in productivity of the recombinant foreign protein was reported in several CHO and BHK cultures which proliferations were arrested by adding nucleotides or nucleosides such as AMP and GMP (Carvalhal et al. 2003), transiently or permanently over-expressing cell-cycle inhibitor p21, p27, p53175P (Fussenegger et al. 1997; Mazur et al. 1998) and lowering cultivation temperature (Kaufmann et al. 2001). Macroscopically, the cell cycle arrest arose from expression of cytostatic gene influences the metabolic activities of host, in which unencumbered by demands for simultaneous production of cellular proteins and all other cellular components, the protein synthesis apparatus and all of the metabolic activity of the cell can in principle be mobilized and devoted entirely to production of the desired recombinant protein in this proliferation-inhibited state (Mazur et al. 1998).

This growth-arrested cultivation mode provided several advantages: slower substrate consumption, higher overall production since the product formation doesn’t compete with other growth-associated metabolic processes for cellular resources (Mazur et al. 1998), overcoming the nutrient and oxygen depletion and accumulation of toxic metabolites caused by uncontrolled cell growth, and it also extended the limited period of
maximum protein production in batch bioreactor (Mazur et al. 1999). To the best of our knowledge, currently this two-stage production of recombinant protein is only restricted in mammalian cells, our current study is to investigate this technology for recombinant protein production in plant cell culture.

1.4.1.1 Chemical and environmental strategies for arresting plant cell cycle

Conventionally, the arrest of plant cell cycle can be achieved by manipulating environmental factors: removal of essential compounds for growth (Menges and Murray 2002) such as phosphate (Amino et al. 1983), sucrose (Riou-Khamlichi et al. 2000) and plant hormones (Nishida et al. 1992); shifting culture temperature; changing light conditions (Hagiwara et al. 2001). However, the synchrony level obtained by environmental strategies is usually low. Therefore, modulating environmental conditions for inhibiting plant cell growth is usually coupled with addition of chemical cell cycle synchronizing reagents. There is strong evidence showing that the use of chemical reagents, usually purine derivatives, like roscovitine (Planchais et al. 1997), indomethacin (Ehsan et al. 1999), and bohemine (Binarova et al. 1998) can achieve high synchronization in plant suspension culture. Such synchronizations exclusively occurred at G1/S or G2/M transition point of cell cycle due to the interaction of chemical inhibitor with specific CDKs. Microtubules, essential for spindle organization and chromosome movement, are dynamic polymers composed of tubulin (Planchais et al. 2000). Antimitotic drugs including colchicines, oryzalin, propyzamide and amiprosphos-methyl (APM) can inhibit polymerization of microtubulin and therefore arrest at the metaphase (Planchais et al. 2000; Verhoeven et al. 1990). In addition, DNA synthesis inhibitors such
as hydroxyurea, aphidicolin (Menges and Murray 2002), mimosine (Perennes et al. 1993; Reichheld et al. 1998) and proteasome inhibitors like MG132 (Genschik et al. 1998) and lactacystin (Lee and Goldberg 1998) were able to arrest cell cycle progression by inhibiting DNA replication and ubiquitin-dependent proteolysis that targets on B-cyclin. However, the drawback of the addition of synchronizing agents is that normally they induce a differential uncoupling of normal synthetic patterns, leading to cell death (Carvalhal et al. 2003; Gong et al. 1995; Sukhorukov et al. 1994).

1.4.1.2. Genetic strategy for arresting plant cell cycle

Genetic method to arrest cell cycle typically involves manipulation of one or more cell cycle regulatory proteins. In CHO and BHK cells, the cell cycle progression was successfully blocked by over-expressing cell cycle inhibitory genes such as cyclin-dependent kinases inhibitors (p27, p21) and tumor repressor genes (IRF-1), respectively (Carvalhal et al. 2000; Carvalhal et al. 2003; Mazur et al. 1999). In plant systems, successful cell cycle arrest by over-expressing a series of cyclin-dependent kinase inhibitors has been demonstrated in Arabidopsis thaliana (Zhou et al. 2002a). Additionally, a negative mutant of plant cyclin dependent kinase CDC2aAtN146 (CDC2a with its Asp146 residue mutated) retains its ability to bind regulatory proteins that are necessary for CDK activity, resulting in a competition between CDC2aAt.N146 and the endogenous CDKs for the same proteins (Veylder et al. 2000). Transgenic tobacco plants that expressed the CDC2aAt.N146 gene displayed a reduced amount of extractable CDK activity. Expression of CDC2aAt.N146 correlated with a decrease in the number of cells resulting from partially arrested cell cycle (Hemerly et al. 1995).
1.4.1.3 Plant cyclin-dependent kinase inhibitor (ICK1)

Among the plant CDK inhibitors, ICK1 is the best characterized cyclin-dependent kinase inhibitor. ICK1 is identified in Arabidopsis thaliana and recombinant ICK1 shows plant kinase inhibitory activity in vitro (Wang et al. 1997). The C-terminal domain of ICK1 contained an important consensus sequence with the mammalian CDK inhibitor p27kip1, and the remainder of the deduced ICK1 sequence showed little similarity to any known CDK inhibitors (Wang et al. 1998). The expression of deletion mutant (deletion of C-terminal 15 or 29 amino acids and N-terminal 108 amino acids) in A. thaliana demonstrated that the conserved C-terminal domain was responsible for in vivo kinase inhibition and N-terminal domain increased the instability in vivo (Zhou et al. 2003). ICK1-GFP fusion protein was shown to localized to nucleus in roots of transgenic plants (Zhou et al. 2003).

By interacting with Cdc2a and three D-type cyclins (D1, D2, D3), over-expression of CaMV 35S-ICK1 in A. thaliana significantly inhibited plant growth, cell division and resulted in loss of plant weight as well. It also modified plant cell morphology such as altered shape of roots, serration of leaves, and enlarged cell size with reduced cell number (Wang et al. 2000). Microinjection of recombinant ICK1 into individual dividing Tradescantia virginiana stamen hair cells caused delay of metaphase transit time(Cleary et al. 2002). Expression of ICK1 in A. thaliana plants and single-celled trichomes showed reduced endoreduplication and cell growth (Schnittger et al. 2003; Zhou et al. 2002a). In addition to ICK1, other CDK inhibitors such as ICK2,
ICK4, ICK5, ICK6, ICK7 and ICKCr have slightly lower, but similar inhibitory effect on plant growth and inhibitory activity against CDKs (Zhou et al. 2002a).

1.5 Research goal and objective

Bioreactor is a potential platform for plant suspension culture to produce high-value recombinant proteins. To improve volumetric productivities of plant cell cultures, it is desirable to operate the culture at high cell density. The continuous cultivation of high-density transgenic tobacco suspension culture will be achieved by using perfusion stirred tank reactor. The online GFP fluorescence will be applied to monitor the culture status in this perfusion culture.

To achieve optimal production of the recombinant protein products, it is important to detect product formation in situ and in real time. Several technical factors (ie. Inner filter effect, culture auto-fluorescence and fluorescence bleaching) have to be considered to accurately estimate GFP or GFP-fusion protein expression in plant cell cultures from online GFP fluorescence intensity. An improved backscatter fluorescence probe model that accounts for the influence of IFE and autofluorescence will be developed for reporting culture GFP concentration from online fluorescence. A state observer will be established using the extended Kalman filter (EKF), based on the fluorescence probe model, a dynamic state model of the plant cell bioreactor. This state observer will provide an attractive means to monitor the dynamic process of the transgenic plant cell culture in real-time.
Considering the great advantages of two-stage production of recombinant protein in mammalian cells (Mazur et al. 1998), in this study, the transgenic *N. tabacum* cell lines that concomitantly carrying both a cyclin dependent kinase inhibitor gene (ICK1, controlled by an estrogen-based promoter) and the CamV35S constitutive promoter-driven reporter gene (GFP) will be established. The estrogen-inducible promoter will be used for a conditional expression of ICK1. This system will be potential for manipulative growth-control of *N. tabacum* suspension to prolong the culture life and enhance the productivity of the foreign recombinant protein (GFP) by arresting the cell division under desirable culture density.

**Reference:**


CHAPTER 2

Production and Online Monitoring of Green Fluorescent Protein in Perfusion Culture of Transgenic Tobacco Cells

ABSTRACT

Long-term (over 45 days) continuous perfusion bioreactor culture of transgenic tobacco cells has been achieved with sustained green fluorescent protein (GFP) production. The culture was maintained at a high biomass concentration (packed cell volume between 40% and 50%) over 30 days. At a perfusion rate of 0.158 vvd and a cell bleed rate of 0.066 vvd, the volumetric productivity of overall GFP (secreted and intracellular GFP) in the perfusion culture is about 35% higher than that in batch culture. This improvement was further enhanced to almost 83% relative to the batch culture, by doubling the cell bleed rate to 0.132 vvd in the perfusion culture. Online GFP culture fluorescence was shown to be able to track the overall trends of cell growth and GFP production in the perfusion culture.

Keywords: green fluorescent protein, perfusion bioreactor, plant cell culture.

2.1 INTRODUCTION

Plant suspension cells cultured in bioreactor are attractive for large-scale production of antibodies, enzymes, and other high-value pharmaceutical and medical proteins. This production system enjoys many unique advantages, including well-controlled culture environment, shorter growth cycle and potentially less susceptible to transgene silencing compared to whole plants (de Wilde et al. 2000), more advanced post-translational modifications than prokaryotic host organisms (Sijmons et al. 1990),
lower cultivation expense than mammalian cell culture, and minimum concern on spreading of genetic modified organisms compared to open field plants (Su and Arias 2003). To improve volumetric productivities of plant cell cultures, it is desirable to operate the culture at high cell density. To this end, perfusion culture presents an attractive means for high-density cultivation. Perfusion culture has long been practiced for the cultivation of hybridoma and mammalian cells. Reports on the use of perfusion bioreactor technology in high-density culture of plant suspension cells have emerged more recently. Culture perfusion has been applied to improve production of plant secondary metabolites such as rosmarinic acid (Su et al. 1993) and berberine (Kim et al. 1991), native secreted proteins and enzymes (Su and Arias 2003; Su and Arias 2003c; Su et al. 1996), as well as high-value recombinant proteins such as hGM-CSF (Lee et al. 2004). An external-loop air-lift perfusion bioreactor was designed and studied for secreted protein production by Anchusa officinalis suspension (Su et al. 1996). A high-density culture (PCV reaching 80%) with increased total extracellular protein concentration (about 2.5 fold higher than batch) was achieved using such air-lift perfusion reactor. More recently, improved secreted acid phosphatase production was successfully achieved using a perfusion stirred tank reactor (Su and Arias 2003; Su and Arias 2003c; Su et al. 1996). In this case, a cylindrical baffle was incorporated into the reactor to form a settling zone, enabling separation of cells and spent media by simple gravitational sedimentation. The stirred-tank design allows enhanced mass transfer and mixing over the air-lift reactor especially under high plant cell concentrations. Cell retention via gravitational sedimentation is simple in its mechanical design and thus less
expensive to implement and less prone to clogging compared with filter-based separation (Su 2000).

Published reports on recombinant protein production in plant cell perfusion-bioreactor cultures remained scarce. To the best of our knowledge, the only published report on recombinant protein production using a perfusion plant cell system was from Lee et al (Lee et al. 2004), on the production of a recombinant human GM-CSF. A 3-9 fold increase in accumulative secreted recombinant hGM-CSF was reported in that study using a stirred-tank bioreactor fitted with a filter for cell/medium separation (Lee et al. 2004). In the present study we employed a perfusion stirred tank reactor equipped with an internal cylindrical baffle (PSTR, similar to that used in (Su and Arias 2003)) to achieve high biomass concentration and improved recombinant protein productivity in suspension culture of transgenic *Nicotiana tabacum* cells expressing a secretory GFP.

The green fluorescent protein is useful as an *in-vivo*, genetically encoded reporter of protein expression, targeting, and cellular activities. In addition to its use in basic sciences, GFP also finds its applications in high-throughput drug screening, development of viral vectors for human gene therapy, and bioprocessing. Previously we have reported the use of GFP in developing an efficient fed-batch plant cell culture process for recombinant protein production (Liu et al. 2001); and by splicing an *Arabidopsis* basic chitinase signal peptide to GFP, we demonstrated efficient secretion of GFP from transgenic tobacco cells and on-line monitoring of GFP fluorescence as a reporter for secreted recombinant protein production in batch plant cell culture (Su et al. 2004b).

The scope of this study is to examine the performance of plant cells cultured in a continuous PSTR for secreted recombinant protein production. Specifically, we
investigated the effect of perfusion initiation timing and cell bleed rate. The recombinant protein productivities under two different cell bleed rates are compared with that of batch cultivation. We also implemented on-line GFP fluorescence to monitor culture status including secreted GFP concentrations, total culture GFP concentrations and cell density in the perfusion bioreactor.

2.2 MATERIALS AND METHODS

2.2.1 Cell culture

Suspension cultures of tobacco cells (*Nicotiana tabacum* cv. Xanthi) transformed with pBIN 1012 *mgfp5* (Scott et al. 1999) was spliced to *Arabidopsis* basic chitinase signal peptide (Hase1off et al. 1997) for GFP secretion (Denecke et al. 1990). Details on plasmid construct and genetic transformation are described elsewhere (Su et al. 2004b). Tobacco cells were maintained in a Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 30 g/L sucrose, 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/L kinetin. Cultures were transferred to a new medium every 8 days. The suspension cultures were maintained at 25°C with shaking at 100 rpm on a gyrorotory shaker.

2.2.2 Culture measurements

Cell dry weight, packed cell volume (PCV) and glucose concentration were determined as previously described (Su et al., 2004). The residual sucrose concentration was measured by using cold anthrone which reacts with the fructose moiety of sucrose after destruction of reducing sugars by strong alkali (Handel 1968). The fructose
concentration was obtained by subtracting glucose concentration from total reducing sugar concentration that was determined by the DNS (3,5-dinitrosalicylic acid) reducing sugar assay (Miller 1959). Cell viability was examined based on fluorescein-diacetate (FDA) uptake (Larkin 1976). GFP extraction from cultured tobacco cells was conducted as previously described (Liu et al., 2001). GFP concentration in the culture was quantified using Western blot and subsequent densitometry analysis against pure GFP standards essentially as described in (Su et al., 2004). Quantification of the immunoreactive GFP bands on the Western blots was done using the Fluor-S MultiImager system and the Quantity-One® image-analysis software (Bio-Rad, CA, USA).

2.2.3 Perfusion bioreactor

The perfusion stirred tank reactor (PSTR) employed in this study is shown in Figure 1 (note that the reactor has a dished bottom). Further details of this reactor can be found in (Su and Arias 2003). Briefly, the glass bioreactor has 3.8 L working volume with a special cylindrical baffle incorporated to form a 600 ml static annular settling zone. The reactor was equipped with a New Brunswick Scientific (Edison, NJ) BioFlo III head-plate. The headspaces of the well-mixing zone and the settling zone are connected via flexible tubing to equilibrate the pressure in the two zones. For agitation, a six-bladed Rushton turbin (on top) and a three-bladed upward pumping marine axial impeller (on bottom, model E-01919-30; Cole Parmer, Vernon Hills, IL, USA) were used. To provide a small amount of mixing without resuspending the cell sediments below the cylinder baffle, we incorporated in the bottom of the bioreactor a magnetic stirring bar rotating at a low speed (about 10 rpm) using a magnetic stir-plate placed beneath the bioreactor jar.
(Corning, model AS635, UK. Figure 1A). Culture aeration was achieved using a sintered glass sparger with 140 μm mean pore size placed between the lower impeller and magnetic stirring bar. The medium perfusion and cell bleed rates were controlled by two ultra-low flow peristaltic pumps (model77: Harvard Apparatus, Holliston, MA).

2.2.4 Operation of perfusion bioreactor

A 7-day-old transgenic *Nicotiana tabacum* culture was inoculated into 3.8 L perfusion bioreactor containing Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 20 g/L sucrose, 1.0 mg/L 2,4-D and 0.1 mg/L kinetin. A polarographic oxygen electrode (Mettler-Toledo Ingold, Bedford, MA, USA) was used to monitor dissolved oxygen. Medium pH was monitored using Mettler-Toledo pH sensor Inpro® 3030 (Mettler-Toledo Ingold, Bedford, MA, USA). Dissolved oxygen was controlled at 30% air saturation under a constant aeration rate of 0.2 vvm by regulating the flow rates of compressed air and pure oxygen via two mass flow controllers (Model MFA1400, Omega Engineering, Stamford, CT, USA) that are regulated using a PID controller (Su et al. 2004b). The cultivation temperature and culture pH were maintained at 25°C and 5.5, respectively, by the BioFlo reactor controller. The initial agitation rate was set at 120 rpm and was increased to 180 rpm after culture PCV reached 50%. Culture foaming was controlled using antifoam C emulsion (Sigma, St. Louis, MO). In the perfusion culture experiment presented here, the reactor was operated under batch mode until day 6, and subsequently the culture was perfused at a constant perfusion rate (0.158 vvd) using fresh MS medium containing Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 20g/L sucrose, 1.0 mg/L 2,4-D and 0.1 mg/L kinetin. Once the culture reached 50% PCV, culture removal was initiated. The cell bleeding was
conducted on a discrete basis (once per 12 hours). The average cell bleed rate was equivalent to 0.066 vvd, and was then raised to 0.132 vvd after day 22. The operation of batch culture was described in (Guan 2003).

2.2.5 On-line monitoring of culture GFP fluorescence

The online GFP fluorescence monitoring system consisting of the light source (100W Xenon lamp), detector, and a bifurcated fiber optic cable attached to a 12-mm diameter fused silica light rod (Fiber Optic Components, Inc, Sterling, MA) was set up identical to that described previously (Su et al. 2005; Su et al. 2004). The internal generator frequency of the Merlin radiometer (Thermal Oriel, Stratford, CT) was equivalent to 30.00. The output of high voltage power supply was set up at 600volts. The excitation light was filtered using a 470 ±10 nm interference filter. The TRACQ32 software (Thermal Oriel, Stratford, CT) was used to acquire and analyze online fluorescence spectra. Online culture fluorescence was reported based on the integral of the emission spectra between 495 and 515nm.

2.3 RESULTS AND DISCUSSION

2.3.1 Cell growth and product formation

In perfusion bioreactor operations, the culture is initially operated under the batch mode for some time before culture perfusion is initiated. From our preliminary study (Guan 2003), we noted GFP production could not be sustained if the perfusion was initiated after the culture entered the stationary phase. In this case GFP production peaked at the early stationary phase and then declined rapidly despite medium perfusion (Guan
2003). Apparently the stationary-phase cells already entered a physiological state that does not support active protein synthesis, and the situation could not be recovered by supplementation of fresh medium and/or removal of potential inhibitory products from the medium by perfusion. To circumvent this problem, here we initiated perfusion during the exponential growth phase of the batch cultivation (Figure 2.2). We also noted from our previous studies (Guan 2003; Su and Arias 2003) that the perfusion culture may be stabilized by bleeding out a portion of the culture. As complete cell retention led to accumulation of dead cells that may impair the culture stability. On the other hand, if the perfusion culture is operated under a very high cell bleed rate, one may not achieve a high biomass concentration and hence defeats the very purpose of perfusion, which is to obtain high-density cultivation. Obviously a balance between the perfusion and bleed rates is crucial in successful operation of a perfusion bioreactor. It should be noted that since perfusion and bleed rates can be controlled separately, perfusion culture with cell bleeding offers greater process flexibility than the chemostats or turbidostats. In the present study, the perfusion bioreactor was conducted at a constant perfusion rate (0.158 vvd) and two different cell bleed rates (0.066 vvd and 0.132 vvd). Due to the low bleed rate and the high biomass solid content in the culture, culture bleeding was done periodically (twice a day as described in 2.2.4). Under these conditions, the transgenic *N. tabacum* suspension cells were cultured in the PSTR over 45 days while the culture perfusate was essentially cell free. With the bleed rate of 0.066 vvd, the cell dry weight and PCV reached 11-13 g/L and 50%-60%, respectively, between days 10 and 21 (Figure 2.2A). When the bleed rate was doubled after day 22, cell density gradually declined and leveled off at a cell dry weight around 9-11 g/L and a PCV around 50%. In a batch
culture operated using the same culture medium, typically we observed the maximum PCV of 50% (Su et al. 2004a). The cell growth profiles in terms of PCV and cell dry weight in the perfusion culture are comparable with each other (Figure 2.2A), indicating similar cellular water content throughout the perfusion culture. The carbon source consumption during the perfusion culture is presented in Figure 2.2C. As in most plant cell cultures, sucrose fed into the medium was rapidly converted into glucose and fructose, with glucose being the preferred substrate. As shown in Figure 2.2 C, the media perfusion was initiated while there were still sufficient amount of residual glucose and fructose (about 3-4 g/L); in addition, carbon source never was limiting throughout the entire culture process, indicated by the presence of residual glucose and fructose in the medium.

Time course of GFP production is depicted in Figure 2.2B. Upon the onset of culture perfusion, both intracellular and extracellular GFP concentrations continued to increase. This was not the case when perfusion was initiated post stationary phase as done in our preliminary study(Guan 2003). Due, in part, to the periodic culture withdraw, the GFP concentration profiles were somewhat jagged during the cultivation. Nonetheless, the intracellular GFP concentrations generally fall between 10-12 mg/L (days 15-26 and day 31 onwards). Note that the intracellular GFP in batch culture typically reached about 12 mg/L (Su et al. 2004a). The drop in GFP concentration seen between days 25-30 was due primarily to doubling the bleed rate. At a bleed rate of 0.066 vvd, extracellular GFP concentration reached and fluctuated around 8 mg/L from day 13; and upon doubling the bleed rate, the extracellular GFP concentration exhibited some degrees of oscillations and stabilized at about 6 mg/L (Figure 2.2B). The overall GFP volumetric productivity $Q_{GFP}$
(mg GFP per culture volume per day) in the perfusion bioreactor (taking into account GFP in both the perfusate and culture bleed) was calculated according to the following equation:

\[ Q_{GFP} = \frac{(D - B) \times P_{EX} + B \times (1 - PCV) \times P_{EX} + B \times P_{IN}}{V} \]

where the intracellular and extracellular GFP concentrations, perfusion rate, bleed rate and bioreactor working volume are designated as \( P_{EX} \) (mg/l), \( P_{IN} \) (mg/l), \( D \) (l/day), \( B \) (l/day) and \( V \) (l), respectively. Considering the high packed biomass volume of plant cell culture, the overall culture GFP concentration, \( P_{overall} \) was calculated as \((1 - PCV) \times P_{EX} + P_{IN}\) (Su et al. 2004b).

Table 2.1. Comparison of GFP productivity in batch and perfusion cultures

<table>
<thead>
<tr>
<th>Cultivation Mode</th>
<th>Batch</th>
<th>Perfusion (bleed rate=0.066vvd)</th>
<th>Perfusion (bleed rate=0.132vvd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volumetric productivity</td>
<td>1.080</td>
<td>1.459</td>
<td>1.971</td>
</tr>
<tr>
<td>(mg / l/ day)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specific productivity</td>
<td>0.142/0.196*</td>
<td>0.127</td>
<td>0.196</td>
</tr>
<tr>
<td>(mg / g cells/day)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Note: * refers to the maximum specific productivity in batch mode.

As shown in Table 2.1, at a perfusion rate of 0.158 vvd and a cell bleed rate of 0.066 vvd, the volumetric productivity of overall GFP (secreted and intracellular GFP) in the perfusion culture is about 35% higher than that in batch culture. This improvement was further enhanced to almost 83% relative to the batch culture, by doubling the cell bleed rate to 0.132 vvd in the perfusion culture. At the lower bleed rate, however, the specific GFP productivity was found to be lower than that of the batch-cultured cells. As the bleed rate was doubled, overall cell growth rate is expected to increase, and we observed a
recovery in the GFP specific productivity. Generally, a key advantage of perfusion culture is its ability to achieve high biomass concentration, with potentially improved product formation from a size-limited bioreactor on a continuous basis (Su 2000; Yang et al. 2000). It is necessary to maintain the cells at a physiological state that supports active protein biosynthesis when the bioreactor is operated at a very high biomass concentration. Results from the present study on the production of GFP and from our previous study on the production of a secreted acid phosphatase (Su and Arias 2003) suggest that plant cells cultured under a high biomass concentration (PCV > 50%) are likely to experience cellular stress that may negatively affect protein biosynthesis, despite the presence of ample amount of nutrient from medium perfusion and the absence of oxygen limitation (dissolved oxygen was controlled at 30% air saturation throughout the perfusion culture). We also observed lower cell viability and specific oxygen demand in the culture under the lower bleed rate. The declined oxygen uptake and reduced viability was also observed in *Anchusa officinalis* perfusion culture under high PCV (Su and Arias 2003). It appears to be beneficial to maintain some level of cell division in the culture (by incorporating culture bleeding) to assure a better recombinant protein production. While it is highly plausible that some level of cell-cycle related events are triggered, it is unclear exactly what molecular events have occurred in cultured plant cells under extremely high biomass concentrations in a quiescent culture. Alternatively, it may be desirable to use a promoter that is particularly active in stationary phase to drive the expression of the target protein product in the quiescent perfusion culture without cell bleeding (Shinmyo et al. 1998). To this end, factors such as increased proteolytic activities in quiescent cultures will need to be characterized to determine the efficacy of such approach.
2.3.2 Online GFP fluorescence monitoring

We have used GFP in a previous study to develop a simple, yet effective nutrient feeding strategy for high-density fed-batch plant cell culture (Liu et al. 2001). Here we examine the use of on-line GFP signals for monitoring continuous perfusion plant cell cultures. In the perfusion culture, cell dry weight was shown to exhibit a trend similar to that of the on-line culture GFP fluorescence (Figure 2.3A). The latter increased linearly with the former during the batch phase of the perfusion culture up to about 8 g/L cell dry weight (Figure 2.3B). Deviation from the linear correlation was noted for data collected beyond the batch phase of the culture. This deviation is believed to result from the inner filter effect associated with high plant cell concentrations. The presence of inner filter effect is further confirmed by analyzing the off-line culture fluorescence of culture samples from the reactor (Liu et al. 2001). High-density culture samples were subject to serial dilution and the fluorescence intensities of the diluted samples did not show linear dependency on the culture concentrations, whereas low-density culture samples did (data not shown).

Similar to monitoring of cell growth, on-line GFP fluorescence was able to track the overall trend of GFP production in the perfusion culture (Figure 2.4) although precise, linear tracking is possible only in the batch phase of the culture. In addition to the inner filter effect mentioned above, the fact that some of the GFP is secreted that is likely to exhibit a different quantum yield than the intracellular GFP, further complicates the monitoring process. Although the precision of GFP online fluorescence as reporter for protein production and cell growth during perfusion condition was somewhat undermined under high cell density compared to its performance in batch counterpart, the accuracy
level observed still warrants GFP fluorescence as a useful tool for tracking the overall trend of GFP production and cell growth in a perfusion plant cell culture. It offers a convenient tool for adjusting the bioreactor operation conditions including the timing for initiating perfusion and culture bleeding, and potentially for adjusting perfusion and bleed rates.

2.4 CONCLUSIONS

In this study we achieved long-term perfusion cultivation of transgenic tobacco cells with improved recombinant protein (GFP) production compared with batch cultivation. It is necessary to initiate culture perfusion before the culture enters the stationary phase, leading to sustained recombinant protein production for a prolong culture period. It is also necessary to incorporate cell bleeding to stabilize the high-density perfusion culture. Since a key advantage of perfusion culture is to operate the culture at high cell density, it remains a challenge to determine the optimal combination of perfusion rate and bleed rate to achieve optimal recombinant protein production in a perfusion reactor. To this end, GFP monitoring which was shown in this study to be able to track the overall trends of recombinant protein production and cell growth in plant cell culture, offers a potential tool to aid on-line adjustment of perfusion and bleed rates. Further experiments however are necessary to validate this potential. In summary, the present study demonstrates the usefulness of continuous perfusion as a means to improve recombinant protein productivity in transgenic plant cell cultures.
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Figure 2.1 (A) Transgenic *N. tabacum* cells cultured in the perfusion stirred tank reactor with on-line sensing of GFP culture fluorescence; (B) Schematic diagram of the perfusion stirred tank reactor.
Figure 2.2 Time course of 7-1 perfusion culture. A. Cell growth B. GFP secretion in spent media and intracellular formation. C. Sugar consumption. “Arrow” indicates the onset of perfusion. The first vertical solid line on day 8 represents the time point that cell bleed (0.066vvd) was started. The second vertical solid line on day 22 represents the time point that cell bleed rate (0.066vvd) was doubled.
Figure 2.3 A. Cell growth monitoring by online GFP culture fluorescence.

B. Plot of Online GFP culture fluorescence v.s. dry cell weight.
Figure 2.4 Overall GFP monitoring by online GFP culture fluorescence.
CHAPTER 3.
Observer-based Online Compensation of Inner Filter Effect in Monitoring Fluorescence of GFP-expressing Plant Cell Cultures

ABSTRACT

The green fluorescent protein (GFP) isolated from the jellyfish *Aequorea victoria* is a very useful reporter for real-time bioprocess sensing. GFP culture fluorescence is a composite signal that can be influenced by factors such as culture autofluorescence, inner filter effect (IFE), and photobleaching. These factors complicate accurate estimation of GFP concentrations from the culture fluorescence. IFE is especially problematic when using GFP in monitoring transgenic plant cell suspension cultures, due to the aggregated nature of the cells and the high biomass concentration in these culture systems. Reported approaches for online compensation of IFE in monitoring culture NADH fluorescence or bioluminescence require online measurement of biomass density or culture turbidity/optical density, in addition to fluorescence/bioluminescence measurement. In this study, culture GFP fluorescence was used successfully to estimate GFP concentration and other important states in bioreactor culture of transgenic tobacco cells, while the influences of IFE and culture autofluorescence were rectified without the need for an additional biomass sensor. This was achieved by setting up a novel model-based state observer. First we developed an improved model for a backscatter fluorescence probe that takes into account the influence of IFE and autofluorescence on reporting culture GFP concentration from online fluorescence. The state observer was then established using the extended Kalman filter (EKF), based on the fluorescence probe model, a dynamic state model of the plant cell bioreactor, and on-line GFP fluorescence measurement. Several versions of the observer were introduced to address practical...
requirements associated with monitoring GFP fluorescence of plant cell cultures. The proposed approach offers an effective means for online compensation of IFE to enable quantitative interpretation of the culture fluorescence signals for accurate reporting of GFP or GFP-fusion protein expression.

Keywords: green fluorescent protein, inner filter effect, plant cell culture, recombinant protein, state estimation.

3.1 INTRODUCTION

Plant cell culture is a potential production platform for high-value recombinant proteins. To achieve optimal production of the recombinant protein products, it is important to detect product formation in situ and in real time. This enables development of efficient process operation/control strategies to improve protein productivity (Baker et al. 2002; Chae et al. 2000). Published studies on process monitoring and control of plant cell cultures are quite scarce. Cloning of the genes encoding the Aequorea victoria green fluorescent protein (GFP) (Prasher et al. 1992) and other GFP-like fluorescent proteins (Verkhusha and Lukyanov 2004) open doors to vast opportunities for developing new process sensing tools to monitor plant cell cultures. Splicing GFP with a broad range of protein partners at either N- or C- terminals has led to development of many functional fusion proteins whose concentration and sometimes even biological activity can be quantitatively correlated with the GFP fluorescence intensity (Albano et al. 1998; Cha et al. 2000; DeLisa et al. 1999; Poppenborg et al. 1997), although in some other cases splicing with GFP or GFP-like proteins may lead to instability of the fusion partners (Cabantous et al. 2005; Tsien 1998). GFP is a relatively small protein (27 kDa) and its fluorescence mechanism is self-contained, requiring no cofactors. Therefore, one can
determine the level of GFP fusion protein on line, in real time, by monitoring GFP fluorescence, which can be achieved using an optical sensor (Randers-Eichhorn et al. 1997; Su et al. 2004). In addition to monitoring recombinant protein products, precise and non-invasive detection of the expression of GFP-based sensor proteins in real time is also highly valuable for studying the dynamics of cellular processes in plant cells. For instance, FRET (fluorescence resonance energy transfer)-based GFP nanosensors have been developed to monitor signal transduction and sugar transport in vivo (Fehr et al. 2002; Miyawaki et al. 1997).

Several technical factors have to be considered to accurately estimate GFP or GFP-fusion protein expression in plant cell cultures from on-line GFP fluorescence intensity. First, both excitation light and emitted fluorescence could be partially absorbed and scattered by cell aggregates or debris. Additional light absorption could take place with certain soluble compounds present in the spent medium (e.g. medium components and secreted metabolites, including GFP). These factors contribute to the so-called “inner filter effect (IFE)” (Lakowicz 1999) that could distort the measured GFP fluorescence signals, leading to erroneous estimation of the actual GFP concentration. In plant cell cultures the IFE deserves particular attention in view of the extensive cell aggregation and the high biomass concentration normally encountered. The IFE encountered during online monitoring of fluorescence intensity of intrinsic fluorophores in microbial and mammalian cell cultures has been the subject of several investigations (Li and Humphrey 1992; Srinivas and Mutharasan 1987; Wang and Simmons 1991). For practical online applications, (Konstantinov et al. 1993) reported a strategy for real-time compensation of the IFE in monitoring the bioluminacent bacterial cultures. The strategy involved
establishing a mathematical model to link the IFE to cell density, and to use an on-line laser turbidity sensor to report the biomass density needed in the calculation of IFE. A similar approach was recently applied to monitor recombinant protein production using bioluminescence in a semiautomated fermentation process (Trezzani et al. 2003). An obvious drawback of such an approach is the requirement of a turbidity sensor in addition to the optical sensor for monitoring culture bioluminescence.

In this study, culture GFP fluorescence measured on line was used successfully to estimate GFP concentration and other important states in bioreactor cultures of transgenic tobacco cells expressing GFP, while the influences of IFE and culture autofluorescence were rectified without the need for an additional biomass sensor. This was achieved by developing a model-based state observer. To enable online monitoring of GFP culture fluorescence, a backscatter optical light-rod system (Su et al. 2004) was used. We have developed an improved backscatter fluorescence probe model that accounts for the influence of IFE and autofluorescence on reporting culture GFP concentration from online fluorescence. The state observer was established using the extended Kalman filter (EKF), based on the fluorescence probe model, a dynamic state model of the plant cell bioreactor, and on-line GFP fluorescence measurement. Incorporation of EKF is expected not only to reduce the measurement noises, it also enables estimation of states that are not readily measurable, partly compensates for uncertain system dynamics (Stephopoulos and Park 1991), and therefore expands the utility of the state/measurement models. While *A. victoria* GFP displays relatively high photostability, other GFP variants and GFP-like proteins are quite susceptible to photobleaching (Shaner et al. 2004). To avoid potential fluorescence photobleaching under prolonged and continuous light excitation (Shaner et
al. 2004; Tsien 1998), it is preferred to measure GFP fluorescence intermittently during culture. To meet this requirement, we have examined several alternative approaches to develop observers that are compatible with intermittent measurement signals. Our proposed approach offers an effective means for online compensation of IFE to enable quantitative interpretation of the culture fluorescence signals for accurate reporting of GFP or GFP-fusion protein expression. This is important not only for optimizing recombinant protein production (Chae et al. 2000; DeLisa et al. 2001), but also for precise real-time monitoring of complex cellular dynamics using fluorescent sensor proteins (Fehr et al. 2002; Griesbeck 2004).

3.2 MATERIALS AND METHODS

3.2.1 Cell Line and Culture Maintenance

Suspension cultures of tobacco cells (Nicotiana tabacum cv Xanthi) transformed with pBIN mgfp5-ER (Haseloff et al. 1997) were used in all experiments. Details on the plasmid construct and genetic transformation are provided elsewhere (Liu et al. 2001). The excitation and emission spectra of the GFP variant (mGFP5) used in this study can be found in the work of (Siemering et al. 1996). mGFP5 exhibits dual excitation peaks at 395 nm and 473 nm of nearly equal amplitude, and a maximum emission peak at 505 nm. Tobacco cells were grown and maintained in a Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 2% glucose, 1.0 mg·L⁻¹ 2,4-D, and 0.1 mg·L⁻¹ kinetin. Cultures were transferred to a new medium every 8 days. The suspension cultures were maintained at 25°C with shaking at 100 rpm on a gyrotory shaker.
3.2.2 Bioreactor Cultures and On-line Sensing of Culture GFP Fluorescence

Bioreactor cultures were conducted in a modified 3-liter stirred-tank bioreactor (BioFlo III, New Brunswick Scientific, Edison, NJ) with on-line monitoring of culture fluorescence and dissolved oxygen control. Aeration, agitation, and temperature were controlled at 0.2 vvm, 150 rpm, and 25°C, respectively. The dissolved oxygen was controlled at 30% air saturation by regulating the composition of sparging gases (air and pure oxygen) using a PID controller as described in (Su et al. 2004). The on-line fluorescence monitoring system, essentially as described in (Su et al. 2004), is shown in Figure 1. The monitoring system consisted of a 100W Xenon lamp light source, a 470 ±10 nm interference filter, a bifurcated fiber optic cable attached to a 12-mm diameter light rod through a removable coupling, a monochromator with a photomultiplier tube, and a digital lock-in radiometer (Merlin model 70100; Thermal Oriel). The Merlin radiometry system drives an optical chopper and acts as a lock-in amplifier to retrieve the signal and reject unmodulated background radiation. Except for the fiber optic cable and the light rod, which were purchased from Fiber Optic Components, Inc. (Sterling, MA), the rest of the fluorescence sensor components were obtained from Thermal Oriel (Stratford, CT). The light rod was inserted into the reactor through the head-plate. Upon excitation with a blue light at 470±10 nm, the resulting culture fluorescence was collected via the light rod in the backscatter mode, and detected via a detector system that consists of a monochromator/photomultiplier tube and the Merlin radiometry system. Fluorescence spectra (495-515 nm) were acquired online and analyzed using the Thermal Oriel TRACQ32 software. In this work, online culture fluorescence was reported based on the peak fluorescence intensity (at ca. 505 nm).
3.2.3 Culture Measurements

Cell dry weight and residual glucose were determined as previously described (Su et al. 2004). GFP extraction from cultured tobacco cells was conducted as described in Liu et al. (2001). GFP concentration in the culture was quantified using Western blot and subsequent densitometry analysis against pure GFP standards essentially as described in Su et al. (2004). Quantification of the immunoreactive GFP bands on the Western blots was done using the Fluor-S MultiImager system and the Quantity-One® image-analysis software (Bio-Rad, CA, USA).

3.3 RESULTS AND DISCUSSIONS

3.3.1 Development of a GFP Fluorescence Measurement Model

The extent of IFE associated with the transgenic tobacco cell culture was characterized by subjecting stationary-phase bioreactor cultures to serial dilution, and the culture GFP fluorescence measured using the backscatter fluorescence sensor. The result from a typical run (in this case based on diluting the culture from reactor run#1) is presented in Figure 3.2. In the culture dilution experiment, the cellular GFP content should remain unchanged at different cell concentrations since they were diluted from the same culture, and as such the nonlinearity between the fluorescence signals and the cell concentrations indicates the existence of the inner filter effect (Srinivas and Mutharasan 1987). Although it was reported that high aeration and agitation rate decreased the fluorescence intensity and augmented fluorescence signal noise (Li et al. 1992), we found that the online GFP fluorescence signal was not affected by aeration and agitation within
typical operating conditions (up to 1 vvm and 300 rpm in the 3-liter BioFlo reactor) for the GFP-expressing tobacco cell culture (data not shown).

Several IFE models exist in the literature that attempted to compensate for IFE when measuring culture fluorescence from intrinsic fluorophores (such as NADH, tryptophan and pyridoxine) in microbial cultures (Srinivas and Mutharasan 1987; Wang and Simmons 1991; Li and Humphrey 1992; (Liden and Niklasson 1993). To our knowledge, there is no published report on modeling IFE associated with fluorescent protein production in plant suspension cultures. Based on the model reported by Wang and Simmons (1991) which was modified from that of Srinivas and Mutharasan (1987), we proposed an improved GFP fluorescence sensing model (for the backscatter probe configuration) that accounts for the IFE by incorporating a modified geometric fraction factor and an error compensation factor. In this model we also take into account the auto-fluorescence caused by cellular compounds (such as pigments and phenolics). To establish the model, we assumed that the attenuation in the excitation radiation and the emitted fluorescence were exclusively due to the absorption and scattering of the cell particles. Here the bulk of GFP is present intracellularly, with negligible amount secreted into the media, due to the inclusion of an ER-retention signal in the construct. The light attenuation is governed by the Beer-Lambert law. We further assumed that the excitation light was a parallel beam perpendicular to the tip of the light rod. Geometric representation of the sensor model is shown in Figure 3.3. On the basis of these assumptions, the fluorescence intensity generated by GFP in the annular control volume consisting of point light sources located at a horizontal distance $x$ from the probe tip and a
vertical distance \( y \) from the centerline of the probe (Figure 3.3) can be expressed as follows:

\[
dF = \phi I_0 e^{-\tau_{ex}C_x} \varepsilon_{ex} C_p 2\pi y \, dy \, dx
\]

where \( \phi \) is the quantum yield of GFP, \( I_0 \) is the excitation light intensity at the tip of the probe, \( \tau_{ex} \) is the turbidity coefficient at the excitation wavelength \( \lambda_{ex} (= 470 \text{ nm}) \), \( C_x \) is the biomass concentration, \( C_p \) represents the average GFP concentration in the culture, \( \varepsilon_{ex} \) is the molar absorption coefficient of GFP at \( \lambda_{ex} \), and \( x \) and \( y \) are coordinates according to Figure 3.3. Given that fluorescence is emitted uniformly in all directions, only a fraction of the emitted fluorescence reached the sensor (light rod) tip surface. In the model of Wang and Simmons (1991), this fraction was considered the same for all point light sources residing on the same cross-section at a distance \( x \) away from the probe tip. This could introduce considerable errors especially when the cross sectional area of the probe tip is large. In the model described here, we consider the fraction of fluorescence detected from a point light source is the same for all point light sources within an infinitely slim annular control volume as depicted in Figure 3.3. The distance between the point light sources within this annular control volume and the center of the probe tip is \( \zeta \) (cf. Figure 3.3A). The portion of emitted fluorescence from the point source reaching the detector can then be expressed as:

\[
f'(x, y, R) = \frac{1}{2\pi} \int_0^\phi \int_0^{2\pi} \frac{x r}{\sqrt{(x^2 + y^2 + r^2 + 2yr \cos \theta)}} \, dr \, d\theta
\]

The development of this geometric fraction factor is described in detail in the Appendix. By taking this geometric fraction factor into account, and by considering absorption/
scattering of emitted fluorescence by cell particles as the emitted light traveled back to the sensor surface, the fluorescence intensity detected from the point light sources within the annular control zone is given by:

\[ dF_0 = 2\pi f'(x, y, R) \phi I_0 e^{-\tau_{em}C_0} e^{-\tau_{ex}C_{ex}} C_p y \, dy \, dx \]  

(3)

where \( \tau_{em} \) is the turbidity coefficient at the emission wavelength. For the tobacco cell culture used here, we found that the values for \( \tau_{em} \) and \( \tau_{ex} \) are essentially the same. Therefore, Eq. (3) is simplified to:

\[ dF_0 = 2\pi f'(x, y, R) \phi I_0 e^{-\tau C_0} e^{-\tau_{ex}C_{ex}} C_p y \, dy \, dx \]  

(4)

where \( \tau = \tau_{ex} = \tau_{em} \) is the average turbidity coefficient. By integrating Eq. (4) for the light path length \( L \), the overall fluorescence intensity detected by the sensor is given by:

\[ F_0 = 2\pi \phi I_0 C_{ex} C_p \int_0^\infty \int_0^\infty f'(x, y, R) e^{-\tau C_0} e^{-\tau_{ex}C_{ex}} y \, dy \, dx \]  

(5)

In order to obtain an approximated analytical expression for \( F_0 \), \( \zeta \) is approximated as \( x \) and the geometric fraction \( f'(x, y, R) \) as \( f(x, R) \) that was reported in Wang and Simmons (1991):

\[ F_{0,t} = 2\pi \phi I_0 C_{ex} C_p \int_0^\infty \int_0^\infty f(x, R) e^{-\tau C_0} e^{-\tau_{ex}C_{ex}} x \, dy \, dx \]  

(6)

where

\[ f(x, R) = \frac{1}{2} \left( 1 - \frac{x}{\sqrt{x^2 + R^2}} \right) \]  

(7)

Wang and Simmons (1991) suggested that \( f(x, R) \) could be approximated by
\[ f(x, R) = \frac{1}{2} \exp(-Sx) \]  

and the parameter, \( S \), is given by:

\[ S = -\frac{1}{x} \ln \left( 1 - \frac{x}{\sqrt{x^2 + R^2}} \right) \]  

which was further approximated using a constant in the work of Wang and Simmons (1991). For the optical light rod system used in the present study, \( S \) can be regarded as a constant with a value of 1.21. With these approximations, Eq. (6) is integrated to obtain the following analytical expression that links the detected fluorescence intensity with the biomass and GFP concentrations:

\[ F_{x,z} = \pi (\phi \cdot I_o \cdot e_{C_p}) \int_{-R}^{R} e^{-2 \pi e_{C_p}} x \, dy \, dx = \frac{1}{2} \pi R^2 \phi \cdot I_o \cdot e_{C_p} \left( 1 - e^{-\left( S + 2 \pi e_{C_p} \right) x} \right) = b_1 C_p \sigma \]  

where

\[ b_1 = \frac{1}{2} \pi R^2 \phi \cdot I_o \cdot e_{C_p} \]  

and

\[ \sigma = \frac{1 - e^{-\frac{S}{\delta}}}{\delta}, \quad \delta = S + 2\pi e_{C_p} \]  

Our numerical simulations indicated that the simplifications (Eqs. (6)–(9)) introduced in the model development may cause considerable errors. We compared the simplified geometric fraction factor \( f(x, R) \) proposed by Wang and Simmons (1991) with the geometric fraction factor we derived \( f'(x, y, R) \) in Eq. (2) and found substantial deviations as shown in Figure 4. This deviation becomes more profound as the point light source gets closer to the probe tip \( (i.e. \text{low } x) \) and/or nearer the outer edge of the light beam \( (i.e. \text{as } y \text{ approaches } R) \). As the dimensionless distance \( x/R \) drops below a threshold,
ca. 3 according to Figure 4, \( f' \) begins to deviate from \( f \). One practical implication from this finding is that as the probe diameter increases, the error introduced in modeling the fluorescence signal by approximating \( f' \) with \( f \) is anticipated to increase. In order to account for the errors brought in by all the simplifications and approximations (Eqs. (6)--(9)), we introduced an error compensation factor \( \eta \) that is denoted as the quotient of Eqs. (5)--(10):

\[
\eta = \frac{4 \int_{0}^{R} \int_{0}^{y(x,y,R)} e^{-\tau C_{x}(x+y)} y \, dy \, dx}{R^{2} \left( 1 - e^{-(S+2\tau C_{x})R} \right)}
\]

The dual integral in Eq. (13) was calculated based on the multiple-segment trapezoidal rule using Matlab (Constantinides and Mostoufi 1999). Using \( S=1.21 \), computer simulations indicated that all these simplifications brought in about 48% error (i.e. \( \eta \approx 0.52 \); Figure 5). Interestingly, we found that this error compensation factor \( \eta \) could be essentially considered as a constant with respect to \( \tau C_{x} \) especially for \( \tau C_{x} \) less than 0.5 (which corresponds to a cell dry weight of 20 g·L\(^{-1} \); Figure 5). By considering the baseline residual fluorescence \( F_{b} \) and cellular autofluorescence (that is subject to similar inner filter effect and is assumed proportional to the biomass concentration), using the simplified fluorescence measurement model (Eq. (10)) and by incorporating the error compensation factor, we arrive at the following IFE model for the plant cell culture expressing GFP:

\[
F = F_{b} + (f_{0}C_{x} + b_{0}C_{p})\sigma \cdot \eta \approx F_{b} + (f_{0}C_{x} + b_{0}C_{p})\sigma
\]
In Eq. (14), $f_0C_x$ and $b_0C_p$ respectively represent the contributions of cellular auto-fluorescence and GFP fluorescence to the overall fluorescence signal. The decaying factor $\sigma$ indicates the extent of attenuation in the online fluorescence signal caused by inner filter effect. Since the parameters $f_0$ and $b_0$ in the IFE model (Eq. (14)) were determined by fitting the experimental data using the Marquardt’s nonlinear least squares method, the approximately 50% constant error was actually absorbed by the fitted values of these two model parameters. This excluded the necessity to use the complex mathematical expression for $\eta$ (Eq. (13)) in the measurement model (Eq. (14)).

To use Eq. (14), a series of experiments were conducted to independently estimate the model parameters: $\tau$, $L$, $f_0$, and $b_0$. First, $\tau$ was determined by measuring fluorescence of a GFP-expressing tobacco cell culture in a cylindrical vessel (which was made entirely black) using the optical light rod system at varying light path-lengths and cell concentrations (by diluting from the same culture). In the measurement set-up, the distance between the tip of the optical light rod and the bottom of the vessel (which is equivalent to the light path length, $L$) was accurately adjusted. With homogeneous culture having uniformed cellular GFP content (i.e., $C_p$ is proportional to $C_x$), $(f_0C_x + b_0C_p)$ can be simplified to $b_0'C_x$. Culture GFP fluorescence $(F - F_b)$ was plotted against the path length at five different cell concentrations (with the highest concentration at 7.4 g·L$^{-1}$). The experimental results along with model fittings (using the Marquardt’s nonlinear least squares method) are presented in Figure 6 which indicates that the IFE model (Eq. (14) with the simplification that $(f_0C_x + b_0C_p) = b_0'C_x$) agrees with the experimental data. From
Figure 3.6, it appears that as the path length exceeds about 3 cm, fluorescence intensities gradually become saturated regardless the cell concentration. This suggests that $L$ could be considered a constant of about 3 cm (independent of cell concentration). Also note that, for $L$ greater than about 3 cm, $\sigma$ value (from Eq. (12)) becomes only a weak function of $L$. Based on the fitting results shown in Figure 3.6, the turbidity coefficient $\tau$ was determined to be 0.025. To determine the parameter $f_o$, wild-type tobacco cells were cultured in a batch bioreactor with time-course measurements of cell concentration and online culture fluorescence. The online acquired culture fluorescence of the wild-type culture is taken as the cellular auto-fluorescence. For the wild-type culture, Eq. (14) becomes:

$$F = F_o + f_oC_s\sigma$$  \hspace{1cm} (15)

Eq. (15) was used to fit the time-course data from the bioreactor experiment with wild-type culture, using predetermined $\tau$ and $S$ values, and $f_o$ was estimated to be $6.083\times10^{-6}$. To determine the parameter $b_o$, a time-course experiment was conducted in a batch reactor with transgenic GFP-expressing culture using an initial glucose concentration of 25 g-L$^{-1}$ (bioreactor run #1). Equation (14) was used to fit the time-course data from this bioreactor experiment using predetermined $\tau$, $S$, and $f_o$ values, and $b_o$ was estimated to be $1.150\times10^{-5}$. The validity of our IFE model was further examined using data from subsequent three bioreactor culture experiments (runs #2~4), having initial glucose concentration at 25, 18.7, and 15.7 g-L$^{-1}$, respectively. As shown in Figure 3.7, using the same set of predetermined model parameters, the measurement model is seen to adequately predict the online culture GFP fluorescence (figure 3.7).
3.3.2 Development of a Dynamic Process Model

To establish the state estimator, a simple unstructured state model is formulated to simulate the batch kinetics of the transgenic tobacco cell cultures. For incorporation into a state estimator, it’s preferred to simplify the state model structure as long as the model can still correctly reflect the overall dynamics of the process (Stephanopoulos and Park 1991). Therefore, the state model was formulated based on simple macroscopic mass balances and assuming unsegregated, balanced growth (Bailey and Ollis 1986). This model simulates the dynamics of cell density \( C_x \), substrate (glucose; \( C_s \)) and product (GFP; \( C_p \)) concentrations:

\[
\begin{bmatrix}
\dot{C}_x \\
\dot{C}_s \\
\dot{C}_p
\end{bmatrix} = 
\begin{bmatrix}
\mu C_x \\
-\mu \left( \frac{1}{Y_{X/S}} - v_B C_s \right) \frac{C_x}{1 - v_B C_x} \\
(k_G \mu + k_N) C_x - k_D C_p
\end{bmatrix} 
+ \begin{bmatrix}
W_x \\
W_s \\
W_p
\end{bmatrix},
\begin{bmatrix}
C_x \\
C_s \\
C_p
\end{bmatrix}_{t=0} = \begin{bmatrix}
C_{x_0} \\
C_{s_0} \\
C_{p_0}
\end{bmatrix}
\]  

(16)

where \( \mu \) is the specific growth rate, \( Y_{X/S} \) is biomass yield coefficient, and \( v_B \) is volume of packed biomass per dry cell weight. This model has accounted for the concentrating effect on substrate concentration due to the considerable increase of biotic-phase volume during plant cell growth. GFP formation is modeled using a Leudeking-Piret type approach that considers both growth-associated as well as non-growth-associated product formation (Luedeking and Piret 1959), and product degradation is assumed to follow a first-order kinetics. \( W_x, W_s \) and \( W_p \) are system noises. The specific growth rate was modeled using the Contois equation (Bailey and Ollis, 1986):
where $\mu_{\text{max}}$ is the maximum specific growth rate, and $B \cdot C_x$ is an apparent saturation constant proportional to cell density and reflects reduction in specific growth rate due to cell crowding. The widely used Monod equation was also tested to model the specific growth rate and was found to give slightly less accurate fitting than with the Contois model (data not shown). It has been widely reported that high biomass concentration attained in plant cell suspension cultures causes so-called “crowding effect”, rendering reduction in the specific growth rates (Su et al. 1993). As such, it seems reasonable that the Contois model gave a better description of the specific growth rate.

Among the model parameters, $\nu_B$, $\mu_{\text{max}}$ and $k_G$ were estimated directly from the time-course data of reactor run #1 (initial glucose concentration $C_{x0}=25.3$ g L$^{-1}$). The other four parameters, $Y_{X:S}$, $B$, $k_N$ and $k_D$ were optimized via multivariable nonlinear least-squares fitting of the model to the data from reactor run #1 using a modified Powell algorithm (Powell 1970). This model fitting process was carried out using the Scientist software (Micromah, Utah). First, the coupled cell growth and substrate consumption model equations (see Eqs. (16) and (17)) were integrated using a fourth-order Runge-Kutta algorithm using the predetermined values for $\nu_B$, $\mu_{\text{max}}$ and $k_G$. By fitting the model simulation to the data from reactor run #1, the values for $Y_{X:S}$ and $B$ were optimized. Subsequently, the values for $k_N$ and $k_D$ were optimized by considering all three dynamic mass balances (for $C_s$, $C_x$, and $C_p$) and by using the predetermined/optimized parameter values for $\nu_B$, $\mu_{\text{max}}$, $k_G$, $Y_{X:S}$ and $B$. The resulting numerical values of the model parameters are summarized in Table 3.1. While literature data for the product formation
model parameters are not readily available, the growth model parameters reported here are in good agreement with the literature data. The values for $\mu_{\text{max}}$ (0.323 d$^{-1}$) and $Y_{X/S}$ (0.474 g·g$^{-1}$) found in this study are very similar to the values reported by (Van Gulik et al. 1992) for *Nicotiana tabacum* cell suspension culture ($\mu_{\text{max}}$ = 0.3264 d$^{-1}$ and $Y_{X/S}$ = 0.57 g·g$^{-1}$). The validity of the dynamic state model was further scrutinized using data from another three independently conducted reactor runs (runs#2~4; each has a different initial glucose concentration and slightly different initial cell density) using the model parameter values shown in Table 3.1. As indicated in Figure 3.8, the model outputs are generally in good agreement with the experimental data, especially for the cell density, $C_x$. Model predictions for $C_p$ and $F$ (calculated from simulated $C_p$ and $C_x$) however show some deviations from the experimental data. In addition, as in all model predictions, model outputs are sensitive to error in the estimates of the initial states since such error is retained in the model output throughout the culture period. This is illustrated in Figure 3.9 and discussed further in the following section. Built on these models, we have incorporated a filter algorithm to develop a state observer to further improve the accuracy of predicting $C_p$ from online culture fluorescence signals, as discussed below.

### 3.3.3 State Observer Development for Tobacco Cell Culture Expressing GFP

A state observer is developed to compensate the IFE based on the state and measurement models described above, and by incorporating the extended Kalman filter. In devising such state observer, it is necessary that the states of interest are observable based on the process measurements. Here, the online culture fluorescence, $F$, is dependent predominantly upon the GFP concentration ($C_p$), whereas the influence of cell density ($C_x$) on $F$ is reflected in the culture autofluorescence and the inner filter effect.
Meanwhile, the glucose concentration ($C_s$) is linked to the cell density via the cell growth model, substrate consumption model and the specific growth rate model. Therefore, all three state variables included in the state model (i.e., $C_x$, $C_s$ and $C_p$) ought to be observable by online measurement of culture GFP fluorescence.

Since only the culture fluorescence intensity ($F$) is used as the measured variable, and by assuming that $F$ is acquired continuously, the measurement equation becomes:

$$y(t) = F(t) = F(t) + V_F(t) = F_0 + (f_0 C_x + b_0 C_p) \sigma + V_F(t)$$

with a measurement covariance $R = Var V_F$. Let $\xi = [C_x, C_s, C_p]^T$ be the state vector, Eqs. (16) and (18) can be respectively expressed as

$$\dot{\xi} = \varphi(\xi) + w; \quad \xi|_{t=0} = \xi_0$$

$$Y = h(\xi) + v$$

where $\varphi(\bullet)$ is the dynamic state function and $h(\bullet)$ is the measurement function, both the system noise $w(= [W_x, W_s, W_p]^T)$ due to modeling error and unknown disturbances and the measurement noise $v(= V_F)$ are assumed to be independent zero-mean white noises.

For continuous measurements, the state observer is expressed as:

$$\dot{\hat{\xi}} = \begin{bmatrix} \dot{\hat{C}_x} \\ \dot{\hat{C}_s} \\ \dot{\hat{C}_p} \end{bmatrix} = \begin{bmatrix} \hat{\mu} \hat{C}_x \\ \hat{\mu} \hat{C}_s \\ (k_G \hat{\mu} + k_N) \hat{C}_x - k_D \hat{C}_p \end{bmatrix} + P C^T R^{-1} (F - F_b - (f_0 \hat{C}_x + b_0 \hat{C}_p) \hat{\sigma})$$

with $[\hat{C}_x, \hat{C}_s, \hat{C}_p]_{i=0} = [\hat{C}_{x0}, \hat{C}_{s0}, \hat{C}_{p0}] = [EC_{x0}, EC_{s0}, EC_{p0}]^T$, and the filtering error covariance matrix $P$ governed by
The filter parameters are listed in Table 3.2. Details on filter parameter tuning have been discussed elsewhere (Li et al. 2003; Su et al. 2003).

The state estimation results are given in Figure 3.8 for four independently conducted reactor runs. The state observer gave a better prediction of $C_p$ than with the model alone. The model was unable to precisely track $C_p$ especially when there was a lag phase at the early culture stage (runs #1 and #4), whereas the state observer was able to track the lag phase with a greater accuracy. Figure 3.9 illustrates the effect of Kalman filtering on reducing the estimation errors resulting from biased initial state measurements. In the presence of a 10% overestimation of either one of the three states ($i.e.$ $\hat{C}_{i0}=0.1 \cdot \hat{C}_{i0}$, where the subscript $i$ corresponds to the state variables $x(C_x)$, $s(C_s)$, or $p(C_p)$), the filter is shown to provide a much more accurate prediction for $C_p$ than using the model alone, judged by the considerably lower normalized mean squared error (Figure 9). Here the normalized mean squared errors for state variable $\xi$ associated with either filtering or modeling take the form of $e_\xi^2=(1/N_s)\sum_i^{N_s}\left(\hat{e}_\xi - \xi_{\max,\text{marmnt}}\right)/\xi_{\max,\text{marmnt}}^2$, where $\hat{e}_\xi$ stands for filtering output or model output of state variable $\xi$, and $\xi_{\max,\text{marmnt}}$ is the maximum value among the $N_s$ measurements. Similar results are obtained with a 10% underestimation ($i.e.$ $\hat{C}_{i0}=-0.1 \cdot \hat{C}_{i0}$). Filter also gives better tracking of the cell density,
although the improvement is not as substantial as that for the GFP concentration (Figure 3.9). Since \( C_s \) is not directly observable from \( F \), estimation error caused by the biased initial state measurements was not reduced in most cases by using the filter; nonetheless note that the estimation error for \( C_s \) is very small when the biased initial estimates are associated with the biomass \( (C_{x0}) \) or product \( (C_{p0}) \) concentrations (Figure 3.9).

Due to concerns on photobleaching of the GFP fluorophore under continuous and extended exposure to light excitation, and consider the slow metabolic rates of plant cells, it is not necessary to monitor the GFP fluorescence continuously. Accordingly, we report two approaches for implementing the EKF with intermittent measurements. The first approach involves the standard two-stage prediction/correction algorithm (Stephanopoulos and Park 1991; Zhang and Su 2002), in which the filtering process consists of two stages: a “prediction” stage in the time interval \( t \in (t_{k-1}, t_k) \) before the time instant \( t = t_k \), and a “correction” stage right at \( t = t_k \). During the “prediction” stage, a continuous prediction of the states \( \xi(t) \) and the estimation error covariance \( P(t) \) is made based on the state model and previous estimation results, \( \hat{\xi}(t_{k-1}) \) and \( P(t_{k-1}) \).

Subsequently in the “correction” stage, a correction is made on \( \hat{\xi}(t_k | t_{k-1}) \) and \( P(t_k | t_{k-1}) \) by means of the EKF and the newly available measurement \( y(t_k) \).

Here we also report an alternative and simplified approach for implementing the EKF that involves a virtual zero-order-holder (ZOH) in the measurement. For each sampling interval \( t \in [t_{k-1}, t_k) \), the measurement signals are assumed constant and equal to the measurement signal \( y(t_{k-1}) \) as if there were a zero-order-holder in the measurement channel, i.e.
\[ y(t) = y(t_{k-1}), \quad t \in [t_{k-1}, t_k) \]  \hspace{1cm} (24)

and hence the online filtering algorithm may take the same forms as that of the continuous-time filter:

\[
\begin{align*}
\dot{\hat{x}} &= \phi(\hat{x}) + \bar{P}C^T\bar{R}^{-1}[y - h(\hat{x})] \\
\dot{\bar{P}} &= \bar{P}A^T + A\bar{P} - \bar{P}C^T\bar{R}^{-1}C\bar{P} + Q
\end{align*}
\]
\hspace{1cm} (25)

where \( \bar{P} \) is the covariance matrix of state estimation error in the ZOH EKF. To obtain a satisfactory filtering result using the ZOH EKF, estimation of the \( \bar{R} \) value can be rationalized as follows. The error introduced by the ZOH approximation on the measurement signals, \( V_{ZOH} \), may be viewed as independent of original measurement noise (which has a variance of \( R \)). If the measurement sampling period \( \Delta \) is not too large and let the average slope of the measured \( F \) curve within the \( i \)th sampling interval \([(i-1)\Delta, i\Delta)\) be \( \beta_i \), then \( V_{ZOH} \) can be considered as to vary linearly from 0 to \( \beta_i \Delta \). Thus,

\[
\bar{R} = R + \text{Var}V_{ZOH} \approx R + \frac{1}{N_s} \sum_{i=1}^{N_s} \int_{(i-1)\Delta}^{i\Delta} \{\beta_i[t-(i-1)\Delta]\}^2 \, dt
\]
\[
= R + \frac{1}{N_s} \sum_{i=1}^{N_s} \left[ \frac{1}{3} (\beta_i \Delta)^2 \right] = R + \frac{1}{3} (\beta \Delta)^2 = k_r (\beta \Delta)^2
\]
\hspace{1cm} (26)

where \( \beta \) is the mean squared root slope of the measured \( F \) curve within \( N_s \) sampling periods, and \( k_r \) is an empirical coefficient that takes into account the effect of the original measurement noise \( R \). From Eq. (26), \( k_r \) should be set greater than 1/3. In our case the mean squared root slope \( \beta \) is approximately \( 2.2 \cdot 10^{-5} \) \( d^{-1} \), and the sampling period \( \Delta \) is set at 0.5 \( d \). From simulations we noted that estimation was not significantly affected when \( k_r \) was varied from 0.4 to 1. Using \( k_r = 0.4 \), the value for \( \bar{R} \) was thus estimated as \( 4.8 \cdot 10^{-11} \),
based on Eq. (26). It can be seen from the above analysis that since the actual fluorescence measurement signals are approximated by a stepwise waveform, a larger measurement error covariance matrix $\bar{R}$ has to be used in general. The longer the sampling interval, the larger the value of $\bar{R}$ should be chosen.

Using these two approaches, the filtering simulation results with intermittent measurements are presented in Figure 3.10. When compared with the filtering results in Figure 3.8 in which fluorescence measurements were assumed to be taken continuously, Figure 3.10 demonstrates that the intermittent filter implementation can also offer fairly good estimation results. For example, in the case of run #3, the normalized mean squared error resulting from the continuous EKF for $C_x (e_x^2)$ and $C_{\rho} (e_{\rho}^2)$ are 0.0021 and 0.0014, respectively (Figure 3.8C), while the two-stage EKF gives $e_x^2 = 0.0024$ and $e_{\rho}^2 = 0.0017$ (Figure 3.10B), and the ZOH EKF gives $e_x^2 = 0.0026$ and $e_{\rho}^2 = 0.0005$ (Figure 3.10D). Both approaches are suitable for practical on-line applications. In the case of prediction/correction approach, the state estimation curves are not very smooth, whereas the ZOH approach provides smoother state estimations and a better overall estimation precision, particularly on $C_{\rho}$. In using the ZOH approach, an equivalent measurement time-lag (corresponding to half sampling period) is introduced. The resulting error on the state estimation could be overcome by raising the value of the measurement variance $\bar{R}$, compared with that used in the two-stage prediction/correction EKF. By increasing $\bar{R}$, however, the ZOH EKF becomes less sensitive to abrupt change in the fluorescence signals.
3.4 CONCLUSIONS

GFP and GFP-like proteins are emerging tools for improving recombinant protein production. These fluorescent proteins could be used to monitor the protein product or the cellular processes relevant to recombinant protein production. In order to use online fluorescence for precise, quantitative reporting, raw fluorescence signals corrupted by interference from the inner filter effect have to be corrected to reflect the true fluorescence. In this work, we developed a novel state observer that effectively compensates for the inner filter effect in real time, without needing a biomass sensor, making accurate estimation of GFP expression in plant cell culture from online culture fluorescence possible. Considering the general nature of the state observer reported here, it could potentially be applied for monitoring other culture systems expressing GFP, GFP variants, GFP-like proteins, or proteins fused to these fluorescent protein tags, using backscatter optical probes, although the optimal observer parameters have to be determined in each individual case.

ACKNOWLEDGEMENT

We thank Dr. Jim Haseloff of the University of Cambridge for providing the pBIN mgfp5-ER construct. This work was supported in part by the United States National Science Foundation (grant no. BES97-12916 and BES01-26191) and the Missouri Food for 21st Century program.
NOTATION

A: partial derivative matrix, Eq. (23)

B: constant in the Contois growth model Eq. (17) (g·g⁻¹)

C: partial derivative matrix for calculating the filtering gain, Eq. (23)

C_p: GFP concentration (mg·L⁻¹)

C_s: glucose concentration (g·L⁻¹)

C_x: biomass concentration (cell dry weight) (g·L⁻¹)

e^2: normalized mean squared error between the filter or model output and the measured value

f: geometric fraction factor reported by Wang and Simmons (1991)

f': modified geometric fraction factor proposed in this work, Eq. (2)

F: culture fluorescence intensity

f₀: auto-fluorescence coefficient in the fluorescence measurement model (g⁻¹·L)

F_b: baseline residual fluorescence

h[•]: measurement function

i, j, k: unit vectors along the rectangular axes x, y, z respectively

K: filtering gain matrix

k_D: rate constant for protein degradation (d⁻¹)

k_G: growth-associated product formation rate constant (mg·g⁻¹)

k_N: non-growth associated product formation rate constant (mg·g⁻¹·d⁻¹)

k_r: empirical coefficient in Eq. (27)

L: light path length (cm)
\( \vec{N} \): normal vector on a surface

\( P \): covariance matrix of state estimation error

\( \bar{P} \): covariance matrix of state estimation error used in Eq. (25) for ZOH approach

\( Q \): covariance matrix of system noise

\( R \): covariance matrix of measurement noise

\( \bar{R} \): covariance matrix of measurement noise used in Eq. (25) for ZOH approach

\( R \): radius of probe tip

\( r \): radius in polar coordinate system on probe tip surface

\( S \): measurement model parameter defined in Eq. (9)

\( t \): time (d)

\( v \): measurement noise vector

\( V_F \): measurement noise of variable \( F \)

\( V_{ZOH} \): measurement noise introduced by the ZOH approximation

\( w \): dynamic noise vector of states

\( W_i \): dynamic noise of state variable \( C_i \)

\( x, y \): rectangular coordinates

\( \tilde{x}, \tilde{y} \): dimensionless rectangular coordinates based on \( R \)

\( y \): measurement vector

\( Y_{\text{YIS}} \): reciprocal of biomass yield coefficient (g·g\(^{-1}\))

\( \beta \): mean squared root slope of the measured fluorescence curve in \( N_s \) sampling periods (d\(^{-1}\))

\( \Delta \): sampling period (d)

\( \delta \): coefficient in the measurement model (g\(^{-1}\)·L) defined in Eq. (12)
$\eta$: error compensation factor defined in Eq. (14)

$\theta$: angle in polar coordinate system on probe tip surface

$\lambda_{em}$: emission wavelength (=505nm)

$\lambda_{ex}$: excitation wavelength (=470nm)

$\mu$: specific growth rate (d$^{-1}$)

$\mu_{max}$: maximum specific growth rate (d$^{-1}$)

$v_b$: packed cell volume per dry wt. (L·g$^{-1}$)

$\xi$: state vector

$\hat{\xi}$: state estimate

$\rho$: radius of a virtual spherical surface $S^o$

$\sigma$: decaying factor due to the inner filter effect defined in Eq. (12)

$\zeta$: distance from the point light source to the center of the probe tip surface

$\tau$: turbidity coefficient (g$^{-1}$·L)

$\tau_{em}$: turbidity coefficient at emission wavelength $\lambda_{em}$ (g$^{-1}$·L)

$\tau_{ex}$: turbidity coefficient at excitation wavelength $\lambda_{ex}$ (g$^{-1}$·L)

$\varphi[\bullet]$: dynamic state function
REFERENCES


Table 3.1. Model parameters

<table>
<thead>
<tr>
<th>Measurement Model</th>
<th>State Model</th>
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<td>$F_b$</td>
<td>$B$</td>
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<tr>
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<td>$k_G$</td>
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<tr>
<td>$L$</td>
<td>$k_N$</td>
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<td></td>
<td>0.368 mg·g$^{-1}$·d$^{-1}$</td>
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<tr>
<td>$S$</td>
<td>$Y_{XS}$</td>
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<tr>
<td>$\tau$</td>
<td>$v_B$</td>
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<tr>
<td>$\mu_{max}$</td>
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<td></td>
<td>0.323 d$^{-1}$</td>
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### Table 3.2. Filter parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<tr>
<td>$\text{Var}X_0$</td>
<td>$0.2 \text{ g}^2\text{L}^{-2}$</td>
</tr>
<tr>
<td>$\text{Var}S_0$</td>
<td>$4 \text{ g}^2\text{L}^{-2}$</td>
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<tr>
<td>$\text{Var}P_0$</td>
<td>$8 \text{ mg}^2\text{L}^{-2}$</td>
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<tr>
<td>$\text{Var}W_X$</td>
<td>$0.1 \text{ g}^2\text{L}^{-2}$</td>
</tr>
<tr>
<td>$\text{Var}W_S$</td>
<td>$2 \text{ g}^2\text{L}^{-2}$</td>
</tr>
<tr>
<td>$\text{Var}W_P$</td>
<td>$4 \text{ mg}^2\text{L}^{-2}$</td>
</tr>
<tr>
<td>$\text{Var}V_F$</td>
<td>$3 \times 10^{-13}$</td>
</tr>
<tr>
<td>$\text{Var}V_F$ (only for ZOH approach)</td>
<td>$2.8 \times 10^{-10}$</td>
</tr>
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Figure 3.1. The GFP fluorescence measurement system with the backscatter light-rod sensor: (1) Xenon lamp housing, (2) excitation filter and chopper assembly, (3) bifurcated optic fiber bundle, (4) power supply for the light source, (5) optical light rod, (6) monochromator, (7) preamplifier, (8) photomultiplier tube, (9) power supply for the photomultiplier tube, (10) Merlin radiometer.
Figure 3.2. The detection of inner filter effect in transgenic *N. tabacum* suspension culture.
Figure 3.3. Geometric representation of the backscatter sensor model: (A) two-dimensional view, (B) three-dimensional view.
Figure 3.4. Comparison between the modified geometric fraction factor \( f'(x, y, R) \) and the geometric factor reported by Wang and Simmons (1991) (i.e. \( f' \) with \( y = 0 \)). Note that in terms of the dimensionless variables, \( \tilde{x} = x / R, \tilde{y} = y / R, \) and \( \tilde{r} = r / R \), Eq. (2) becomes

\[
f'(x, y, R) = \frac{1}{2\pi} \int_0^{2\pi} \frac{\tilde{x} \tilde{r}}{\left( \sqrt{\tilde{x}^2 + \tilde{y}^2 + \tilde{r}^2 + 2\tilde{y} \tilde{r} \cos \theta} \right)^2} d\tilde{r} d\theta.
\]
Figure 3.5. The error compensation factor $\eta$ as a function of $\tau C_x$ under different light path lengths; note that $R = 0.6$ cm.
Figure 3.6. Determination of turbidity coefficient $\tau$ and $S$ in the fluorescence measurement model. GFP fluorescence of undiluted culture at a cell dry weight of 7.4 g L$^{-1}$ ($\circ$), culture at 80% original cell density ($\square$), at 60% original cell density ($\diamond$), at 40% original cell density ($\triangle$), and at 20% original cell density ($\times$), as a function of light path length. The curves represent model fitting using a simplified version of Eq. (14) (see text for detail).
Figure 3.7. Estimation of culture GFP fluorescence using the measurement model ($F$ as a function of $P$ and $X$). (A) run #1, (B) run #2, (C) run #3, (D) run #4. Experimental data (○), model fitting (---).
Figure 3.8. Filtering result based on continuous $F$ measurement. (A) run #1, (B) run #2, (C) run #3, (D) run #4. Experimental data ($\circ$), model output (---), filtering output (—).
Figure 3.9. Effect of filtering on reducing estimation errors resulting from erroneous initial state values (data from run #1).
Figure 3.10. Filtering result based on intermittent $F$ measurement; data from runs #1 ((A) and (C)), and #3 ((B) and (D)). (A) and (B): prediction/correction approach; (C) and (D): zero-order-hold approach. Legend: experimental data (○), model output (---), filtering output (—).
CHAPTER 4. Cell Cycle Engineering Of Transgenic Plant Suspension Culture

ABSTRACT

Controlled-proliferation by genetically arresting cell cycle progression has been shown to increase the production of heterologous recombinant proteins in mammalian cell cultures. In this study, we examine the applicability of such approach in plant cell cultures. We constructed transgenic *Nicotiana tabacum* cell lines that concomitantly carry a cyclin dependent kinase inhibitor gene (*Arabidopsis* ICK1, controlled by an estrogen-inducible XVE promoter) and a reporter gene (GFP, driven by the constitutive CaMV35S promoter). The proliferation of ICK1-expressing cell suspension culture was essentially arrested, with a specific growth rate two fold lower than that of proliferation-competent control cell lines. Flow cytometry analysis of the ICK-1 expressing cells revealed a high degree of cell cycle arrest at G1/S, demonstrating the effectiveness of inducible ICK-1 expression in controlled proliferation of culture plant cells. However, in contrast to the mammalian systems, the recombinant reporter protein production was not improved as a result of ICK-1 expression. About 3-fold decrease in specific productivity of GFP was observed upon expression of ICK1. A quantitative RT-PCR analysis showed decreased GFP transcript upon ICK-1 induction, suggesting the 35S promoter may be suboptimal at G1/S. On the other hand, we noted reduced viability, total protein synthesis, and glucose consumption in the ICK1-expressing cultures, indicating that ICK1-mediated cell-cycle arrest in *N. tabacum* suspension culture negatively affected cellular metabolic activity and protein synthesis. These data suggest the reduced GFP productivity from ICK-1
expression may result from both decreased metabolic activity and cell-cycle dependency of the CaMV 35S promoter.

**Key Words:** Cell cycle arrest, green fluorescent protein, cyclin dependent kinase inhibitor, estrogen-inducible promoter, plant cell culture

**4.1 INTRODUCTION**

Plant cell suspension cultures have been used as an essential and important host for production of a large number of pharmaceutical proteins or plant metabolites due to the advantages they offer, such as eukaryotic post-translational modifications over microbial cultures and cost effectiveness over mammalian cells cultivation (Sijmons et al. 1990; Simmons et al. 1991; Su et al. 1996). Furthermore, the production of high-value pharmaceutical recombinant protein by fermentation of plant suspension culture in bioreactor can circumvent problems such as environmental variation, insect disease, and heterogeneous properties of crops associated with the traditional agricultures. By targeting the recombinant protein to the secretory machinery of the plant cells, the recombinant protein downstream recovery from plant suspension cells become easier and more cost effective than that from whole plants. Currently, there exists an interest to enhance the productivity of recombinant protein in plant suspension culture.

To achieve a high productivity of foreign recombinant protein, a novel two-stage production process was first proposed in Chinese hamster ovary (CHO) cell lines by (Fussenegger et al. 1997). In such a bioprocess, a proliferation phase leads to a desired cell density, followed by an extended production phase during which the cells remain
growth-arrested (Mazur et al. 1998). In the past few years, this proliferation-controlled technology has been widely applied in a various bioprocesses. The production of secreted alkaline phosphatase (SEAP) controlled by a tetracycline-repressive promoter was 10-15 fold improved in CHO cells over-expressing cell-cycle inhibitor p21, p27, p53175P on a bi-cistronic vector (Mazur et al. 1998). Such an increase in the productivity of recombinant foreign protein was also observed in several CHO and BHK cultures which cell cycle progression was arrested by adding nucleotides or nucleosides such as AMP and GMP (Carvalhal et al. 2003), over-expressing transcription factor IRF-1 (Carvalhal et al. 2000) and lowering cultivation temperature (Kaufmann et al. 2001). Even though such an enhancement in recombinant protein productivity was conceived as the consequence that the metabolic activity/cellular apparatus for cell division is relieved to recombinant protein production (Mazur et al. 1998), the precise mechanism still remains unclear.

Conventionally, the arrest of plant cell cycle can be achieved by removal of required compounds for growth (Menges and Murray 2002) such as phosphate (Amino et al. 1983), sucrose (Riou-Khamlichi et al. 2000) and plant hormones (Nishida et al. 1992). Additionally, manipulating environmental factors such as shifting culture temperature and changing light conditions (Hagiwara et al. 2001) will also lead to cell cycle block. The use of chemical reagents also shows strong evidence to synchronize plant cell cycle. The most commonly used chemical cell-cycle inhibitors include purine derivatives, such as roscovitine (Plancais et al. 1997), indomethacin (Ehsan et al. 1999), and bohemine (Biranova et al. 1998) and DNA synthesis inhibitors such as HU, aphidicolin (Menges and Murray 2002) and mimosine (Perennes et al. 1993; Reichheld et al. 1998). However, the drawback of environmental or chemical strategy is that they normally induce an
undesirable side effect or even differential uncoupling of normal synthetic patterns, leading to cell death (Carvalhal et al. 2003; Gong et al. 1995; Sukhorukov et al. 1994).

To this end, a genetic strategy drew more and more attention in plant cell cycle regulation. The cell cycle regulation mechanism is highly conserved within all eukaryotes. The cyclin-dependent kinases (CDKs) are specific serine/threonine kinases that control progression through the cell cycle. Their activity is regulated both by association with cyclin regulatory subunits and by specific phosphorylation and dephosphorylation events (Huntley and Murray 1999). The genetic method to arrest plant cell cycle is principally achieved by modulating the activity of CDKs-cyclin complexes. A family of plant cell cycle regulator (CDK inhibitor), ICKs, has shown to inhibit plant CDK activity in vitro. As the first identified CDK inhibitor in *A. thaliana*, ICK1 had strong interaction with Cdc2a/CDKA and three D-type cyclins (D1, D2, D3), hence resulted in an accumulation of cells at G1 phase. Over-expression of CaMV 35S-ICK1 in *A. thaliana* significantly inhibited plant growth, cell division and resulted in loss of plant weight. It also modified plant cell morphology such as shape of roots, serration of leaves and enlarged cell size (Wang et al. 2000). Microinjection of recombinant ICK1 into individual dividing *Tradescantia virginiana* stamen hair cells caused a delay in metaphase transit time (Cleary et al. 2002). Expression of ICK1 in *A. thaliana* plants and single-celled trichomes showed reduced endoreduplication (Schnittger et al. 2003; Zhou et al. 2002) and cell growth.

In this study, we took advantage of the already-available in our lab transgenic *N. tabacum* cell lines and plants which constitutively express a secretory mGFP5 construct spliced to *Arabidopsis* chitinase signal peptide as a model. By subsequently transforming
the above-mentioned *N. tabacum* cell lines with an estrogen-inducible *Arabidopsis* cyclin-dependent kinase inhibitor (ICK1), we obtained the transgenic *N. tabacum* cell lines co-expressing a reporter gene (GFP) and a cell cycle regulator gene (ICK1) under the control of different promoters. The aim of this study was to examine the effects of over-expressing the *Arabidopsis* cyclin dependent kinase inhibitor (ICK1) on (1) cell cycle arrest of *N. tabacum* suspension culture; and (2) production of foreign recombinant protein (GFP).

4.2 MATERIALS AND METHODS:

4.2.1 Plasmid constructions

The basic binary vector pER8 for the estradiol-inducible system was converted into the Gateway®-compatible binary vector pXVE-attB by introduction of the Gateway® attB cassette under the inducible promoter by blunt-end ligation at the Klenow-filled *XhoI* site. A 6xHistidine (6xHis) tag was introduced by PCR at the N-terminus of the *A. thaliana* ICK1 cDNA (provided by Prof. Larry Fowke of the University of Saskatchewan, Canada) and the PCR product was first subcloned into the pCR2.1-TOPO cloning vector (Invitrogen, CA, USA) and then subsequently transferred as an *EcoRI* fragment into the pENTR1A vector (Invitrogen, CA, USA) from which the *EcoRI* fragment containing the ccdB gene was removed, to generate a Gateway ICK1 entry clone. After a Gateway® LR clonase™-mediated recombination, a plant transformation vector carrying the 6xHis-tagged ICK1 cDNA under the XVE promoter
and a hygromycin resistance cassette was established as mapped in figure 4.1 and confirmed by sequencing.

4.2.2 Agrobacteria-mediated transformation and selection

Transformation of *Nicotiana tabacum* was conducted by an *Agrobacterium tumefaciens* -mediated method as described by (Fisher and Guiltinan 1995) with minor modifications. Leaf discs from the GFP-expressing tobacco leaf discs (cell line# 12-9)/GFP-SEAP expressing tobacco leaf discs (Cell line# 10-6) were inoculated *A. tumefaciens* strains LBA 4404 or C58C1 cells carrying the pXVE-ICK1-N-6his binary vector for 3 days at 22 °C, 16h-day/8h-night photoperiod on plates with solid Murashige & Skoog (MS) basal medium (Murashige and Skoog 1962). The thoroughly-rinsed leaf discs were then transferred on selective MS plates containing 1 mg/L BAP (6-Benzyaminopurine), 35 mg/L hygromycin B, 300 mg/L kanamycin, and 500 mg/L cefotaxime for transgenic calli- and shoots regeneration. Plates were refreshed every 10 days. Four-week old transgenic shoots were transferred onto M.S. basal media with 35 mg/L hygromycin B, 300 mg/L kanamycin, and 500 mg/L cefotaxime for rooting.

4.2.3 Generation and maintenance of transgenic *N. tabacum* cell culture

Stem tissue from young primary transformants was cut into small pieces and positioned on solid Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 30 g/L glucose, 1.0 mg·L⁻¹ 2,4-D, 0.1 mg·L⁻¹ kinetin, 500 mg/L carbenicillin and 35 mg/L hygromycin for callus induction. 7-day old suspension cells were subcultured with 15-20% (v/v) inoculation size to 200ml cultures in 500ml flask.
Tobacco cells were grown and maintained in a Murashige and Skoog medium supplemented with 30 g/L glucose, 1.0 mg·L⁻¹ 2,4-D, and 0.1 mg·L⁻¹ kinetin. The suspension cultures were maintained at 25°C with shaking at 100 rpm on a gyrotory shaker. For time course study of transgenic ICK1 expressing suspension culture, induction of ICK1 expression was conducted by adding 20 mg/L (final concentration) 17-\(\beta\)-estradiol (dissolved in 100% ethanol) at the beginning of time course.

### 4.2.4 Genomic DNA isolation and PCR (polymerase chain reaction)

The Genomic DNA was isolated from transformants and wild-type tobacco by using Wizard genomic DNA purification kit (Promega, Madison, WI, USA). Basically, 40 mg leaf powder was added to 600 μl nuclei lysis solution and incubated at 65°C for 15 min. 3 μl of Rnase solution was added to each reaction and incubated at 37°C for 15 min. Cool sample to room temperature, and 200 μl protein precipitation solution was added and vortexed at high speed for 20 seconds. After centrifugation at 13,200 g for 3 min, supernatant was transferred into a clean 1.5 ml centrifuge tube with 600 μl isopropanol. The genomic DNA was precipitated out after 10 min incubation. Subsequent to centrifugation at 12,000×g for 10 min, the pellets were washed with 70% ethanol. Air-dried DNA pellets were redissolved in 100 μl rehydration solution. The incorporation of ICK1 gene into transformants' genome was verified by PCR using gene-specific primers.
4.2.5 Isolation of total RNA

Total RNA was isolated using TRIZOL reagent (Gibco BRL) according to manufacture's protocol. 500 mg of plant leaf tissues were grinded in liquid nitrogen and homogenized in 0.5 ml TRIZOL reagent for 5 min. Then, 0.2 ml chloroform was added per 0.5 ml TRIZOL. The samples were shaken vigorously by hand for 15 seconds and incubate at room temperature for 3 min. After centrifugation at 12,000×g at 4 °C for 15 min, the aqueous upper-phase was transferred to a new tube with 0.6 ml pre-chilled isopropanol. RNA was precipitated after incubation at -20°C for 10 min. Subsequent to centrifugation at 12,000×g for 10 min, the pellets were washed with 70% ethanol. Air-dried RNA pellets were redissolved in 50 μl DEPC H₂O. RNA concentration and purity were determined photometrically at 260/280 nm. The RNA was resolved and examined by 1% formaldehyde- containing agarose gel.

4.2.6 RT-PCR (reverse transcription-polymerase chain reaction)

Reverse transcription reaction was conducted by using SuperScript™ III (Invitrogen, CA, USA). Briefly, 1 μg DnaseI-treated total RNA (RQI Rnase-free Dnase, Promega, USA) dissolved in 13 μl DEPC water was heat shocked at 65°C for 5 mins with the presence of 1 μl 50 μM oligo dT20 and 0.5 μl 10 mM dNTP. After chilled on ice for 1 min, 0.5 μl SuperScript™ III reverse transcriptase (200 units/μl), 1 μl 0.1M DTT and 4 μl 5×First Strand Buffer (Invitrogen, CA, USA) were added to reaction mix, incubated at
50°C for 1 hr and inactivated at 75°C for 15 mins. The synthesized cDNA (amount based on 100 ng initial RNA) was used as a template for PCR amplification.

4.2.7 Quantitative RT-PCR (reverse transcription-polymerase chain reaction)

To quantify and compare the transcriptomes of ICK1 in different transgenic cell lines, a two-step quantitative RT-PCR was applied based on Comparative C_T Method (Relative Quantification of Gene Expression, User Bulletin #2, Applied Biosystems, CA, USA).

To standardize the amount of sample RNA or cDNA loaded to each reaction, an endogenous control (Nicotiana tabacum β-actin) was amplified separately from ICK1 amplification. The synthesis of cDNA referred to section 4.2.5. The amplified PCR products were labeled with iQ™ SYBR green supermix (Bio-Rad, CA, USA) and detected by iCycler iQ Real-Time Detection System (Bio-Rad, CA, USA). The real-time PCR conditions were listed in table 4.1. Three pairs of primers possess different annealing sites on ICK1 and one pair of primers for Nicotiana tabacum β-actin were designed. ICK1 primerA: GTGAGAAAATATAGAAAAGCTAAAGG as a forward primer and CTCTAACTTTACCCATTCGTAACGTCC as a reverse primer.

ICK1 primerB: GTATCGACGGGGTACGAAGA as a forward primer and GGCATCTCCGTCATCAATT as a reverse primer.

ICK1 primerC: GTATCGACGGGGTACGAAGA as a forward primer and TCA TCAAA TTCGCTTCTCC as a reverse primer.

Actin: AAGCTGTGTGTGCCCTATAC forward and TCCAACTCTTTGCTCATAGTC reverse primers.
4.2.8 In vitro Kinase Assay

As described by (Liu et al. 2001), 1 mg suspension cells were collected and homogenized in 2 ml extraction buffer [25 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM NaF, 1 mM Na$_3$VO$_4$, 1 mM β-glycerophosphate, 2.5 mM EDTA, 400 μg/ml AEBSF {(4-(2-aminoethyl)-benzenesulfonyl fluoride}), 1 μg/ml leupeptin and 1 μg/ml pepstatin] by ultrasonication (Wang et al. 1998; Zhou et al. 2003). 200 μg total protein was pulled down by 20μl p13$^{suc1}$-agarose beads (Upstate, Waltham, MA, USA). After 2 h incubation at 4°C, the p13$^{suc1}$-agarose beads mixtures were washed three times by using washing buffer containing 50 mM Tris pH 7.4, 250 mM NaCl, 0.1% Triton-100, 2 mM EDTA, 1 mM DTT, 10 μg/ml antipain, 10 μg/ml soybean trypsin inhibitor, 10 mM β-glycerophosphate, 1 mM NaF and 0.2 mM Na$_3$VO$_4$. (Wang et al. 1998). The p13$^{suc1}$-agarose beads complexes were resuspended in 10 μl assay dilution buffer I (ADBI) containing 20 mM MOPS (pH 7.2), 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM Na$_3$VO$_4$, 1 mM dithiothreitol (Upstate, Waltham, MA, USA). 10 μl magnesium/ATP cocktail (75 mM MgCl$_2$ and 500 μM ATP in ADBI), 10 μl cdc2 substrate cocktail(2 mg/ml Histone H1), 10 μl kinase inhibitor cocktail (20 μM PKC inhibitor peptide, 2 μM PKA inhibitor peptide, 2 μM R24571 in ADBI) and 10 μl ADBI (Upstate, Waltham, MA, USA) was added for each reaction respectively. After 30 min incubation at 30°C, the p13$^{suc1}$-agarose beads were pelleted down and 40 μl supernantant were loaded onto SDS-PAGE (12.5% gel). The detection of phosphorylated Histone H1 was conducted by
polyclonal anti-phospho-histone H1 (Upstate, Waltham, MA, USA) as described by manufacture’s protocol.

4.2.9 Flow Cytometry Analysis

For nuclei preparation, 1 g (fresh weight) of transgenic Nicotiana Tabacum cells were resuspended in 10 ml enzyme cocktail (1% cellulase RS, 0.1% pectolyase Y23, 0.4 M mannitol buffer, pH6.0) and treated for 1 hour with 50 rpm shaking. The Nicotiana Tabacum protoplasts were pelleted after centrifuging the enzyme-treated sample for 8 min at 2500 rpm. The Nicotiana Tabacum protoplasts were resuspended in 10 ml nuclei isolation buffer (Darzynkiewicz et al. 1994). The nuclei were released by passing the protoplasts through 25 gauge syringe needle 3 times. The nuclei suspensions were filtered by using 20 micron polycarbonate membrane (OSMONICS INC., MN, USA). The nuclei were collected by centrifugation at 3000 rpm for 8 min and resuspended in 0.5-1 ml nuclei storage buffer (Darzynkiewicz et al. 1994). The nuclei were frozen at -80°C for flow cytometry analysis.

The isolated nuclei were pre-treated with Rnase A and Rnase B (0.4 mg/ml final concentration for each Rnase) at room temperature for 1 hr. The Rnase-treated nuclei were stained by mixing with 100 μg/ml propidium iodide (1v:1v) for 30 min. The nuclei counting and cell cycle analysis were conducted by using a single-laser based flow-cytometer (Beckman-Coulter XL, CA, USA). The histogram analysis was conducted by software FlowJo (Macintosh 6.2).
4.2.10 In situ cell death detection

The In situ cell death detection was performed by referring to (Zhang et al. 2003) with minor modifications. 0.5 g fresh weight of cells were washed twice by PBS (pH 7.4) and fixed with 1 ml 4% paraformaldehyde in PBS (pH 7.4) for 30 mins at room temperature. The fixed cells were rinsed once by PBS (pH 7.4) and followed by 6 min permeabilization in 1 ml 0.1% Triton X-100 in 0.1% sodium citrate on ice. After washed once by PBS (pH 7.4), cells were incubated with TUNEL reaction mixture following the manufacture’s instruction (In Situ Cell Detection Kit, TMR red. Roche Applied Science, Indianapolis, IN, USA). The N. tabacum cells treated by 100 mM H₂O₂ overnight were included as positive control (Houot et al. 2001). The cells were examined under fluorescence microscope (OLYMPUS BX-60).

4.2.11 Other culture measurements

Cell dry weight, packed cell volume and residual glucose were determined as previously described (Su et al. 2004). GFP extraction from cultured tobacco cells was conducted as described in Liu et al. (2001). GFP concentration in the culture was quantified using Western blot and subsequent densitometry analysis against pure GFP standards essentially as described in Su et al. (2004). Quantification of the immunoreactive GFP bands on the Western blots was done using the Fluor-S MultiImager system and the Quantity-One® image-analysis. Total soluble protein concentration was determined by protein assay reagent (Bio-rad, Hercules, CA) with bovine serum albumin as standards (Bradford 1976).
4.3 RESULTS AND DISCUSSION

4.3.1 Genetic characterization of selected transformants

4.3.1.1 Detection of ICK1 in transgenic *N. tabacum* genome and β-estradiol inducible ICK1 expression

The leaf tissue of hygromycin-resistant *N. Tabacum* plants regenerated from agrobacteria-mediated transformation were used to isolate genomic DNA as PCR templates. The PCR amplicons were examined on 1% agarose electrophoresis (shown as figure 4.2). Totally, 29 explants were tested. Due to the regenerated *N. Tabacum* plants were selected on a 17% higher hygromycin B selective media than that of other studies (Penaloza-Vazquez et al. 1995), ICK1-PCR positive were achieved in all of the 29 tested cell lines. The ICK1 transcription was assessed by using gel-based RT-PCR. 20 three-week explants were transferred into fresh M.S. media in the absence or presence of 20 mg/L β-estradiol for 40 hrs. To generate cDNA, total RNA were isolated from both the induced transgenic *N. tabacum* leaf tissue and its counterpart without induction. As shown in figure 4.3, all the tested cell lines had detectable ICK1 transcripts even without adding inducers, indicating a leaky expression of estrogen-inducible promoter XVE. Additional agrobacteria-infiltration-mediated transient expression with XVE-GFP constructs in the wild-type background (data not shown) also yielded detectable protein levels without the addition of the inducer gave further support to the hypothesis that the detected ICK1 mRNA levels are in result of a basal activity of the XVE system in *N. tabacum*, rather than cross-reactivity of the ICK1 primers used in the PCR reactions. The XVE promoter was first proposed as a tightly regulated and highly inducible promoter in
Arabidopsis and tobacco transgenic plants by (Zuo et al. 2000). Our observation does not agree with the previous reports about XVE system. One explanation for the discrepancy may lay in the different levels of sensitivity of the detection methods used. We used RT-PCR in our study, but Zuo et al (2000) used Northern blot assay. The leaky expression might be caused by the endogenous phytoestrogens (e.g. β-sitosterol) in N. tabacum, while Arabidopsis is known to be a low phytoestrogen-producing plant. Tightly-controlled inducible promoter is a prerequisite for construction of growth-controlled cell lines as even slight expression of anti-proliferation genes would lead to growth arrest and counter-selection of desired clones (Mazur et al. 1998; Meents et al. 2002). Although the A. thaliana protoplast culture expressing a dominant negative CDC2aAt gene showed reduced CDKa kinase activity, it was demonstrated incompetent to block cell division in A. thaliana protoplast culture. The low inducible levels of CDC2aAt.N146 suggested that a counter-selection against strong inducible lines had occurred (De Veylder et al. 2000). Such a counter-selection was probably due to the leaky expression of a dominant negative CDC2aAt gene that was controlled by tetracycline-inducible Triple-Op promoter. Hence, there is a possibility that the selected clones in this study could also be poor ICK1-producing cell lines due to the counter-selection. To answer such a question, a quantitative RT-PCR was conducted to quantify the transcription level of the transgenic N. tabacum cell lines.

4.3.1.2 Measurement of ICK1 expression by quantitative RT-PCR

4.3.1.2.1 Validation of quantitative RT-PCR experiment conditions

A quantitative RT-PCR was conducted to screen for higher ICK1 expression cell lines. The leaky expression level of ICK1 controlled by XVE promoter was also
determined. To establish a sensitive and reliable quantitative RT-PCR method, the optimizations for each stage including RT-Reaction, primer design, reference gene (previously denoted as housekeeping gene), real-time PCR and data analysis are absolutely necessary (Bustin and Nolan 2004).

The primers for both ICK1 cDNA and *Nicotiana Tabacum* β-actin amplifications were first investigated in terms of data reproducibility among different primers with different annealing sites and whether those primers form significant amount of primer dimmers. As shown in figure 4.4 A, the three pairs of ICK1 primers named as ICK1A, ICK1B and ICK1C gave a high reproducible C_T (CT,A=22.4,CT,B=22.6,CT,C=22.5), which demonstrated the real-time amplification of ICK1 is not primer sequence dependent. According to the single and fine peak showed up at approximately 85°C for both ICK1 and β-actin on the melting curve (figure 4.4 B), all of the primers (ICK1-A, ICK1-B, ICK1-C, *Nicotiana Tabacum* β-actin primers) do not form interfering primer dimmers. Additionally, the agarose gel-electrophoresis of the end products from real-time PCR did not observe any non-specific band except 245-bp ICK1 amplicons (data not shown). The parallel amplification curves between gene of interest and internal control would most likely ensure a relative equal amplification between ICK1 and *Nicotiana Tabacum* β-actin. Therefore, primers ICK1-B were chosen for ICK1 cDNA amplification in real-time PCR.

The resolving power of RT-PCR is also limited by the efficiency of RNA to cDNA conversion, which mainly depends on the enzyme used (Bustin and Nolan 2004). The efficiency of RT-PCR was investigated by gradually increasing amount of RNA (500 ng, 1 µg, 2 µg) under fixed amount of SuperScript™ III (100 units) as described in section 4.2.5. No significant variations were found in these conditions (ΔC_T,500ng RNA = -
5.35 ± 0.21, \( \Delta C_{T,1\mu g\ RNA} = -5.30 \pm 0.14 \), \( \Delta C_{T,2\mu g\ RNA} = -5.65 \pm 0.07 \). To use the comparative C\(_T\) method, the relative efficiency of target (ICK1) gene and reference gene (\textit{Nicotiana Tabacum} \( \beta \)-actin) and optimal initial cDNA added to each amplification were required to be tested under a series of folds dilutions in initial cDNA (presented in forms of total RNA amount used in Reverse-transcription reaction). As shown in figure 4.5, the amplification signal – cycle number C\(_T\) of both ICK1 and \textit{Nicotiana Tabacum} \( \beta \)-actin showed a linear dynamic response (\( R^2_{\text{ICK1}} = 0.98 \) and \( R^2_{\text{actin}} = 0.98 \)) to a series of initial cDNA dilutions (represented in forms of log 50 ng, log 100 ng, log 200 ng, log 400 ng total RNA). The validity of comparative C\(_T\) method depends on whether the efficiency of the target amplification and the efficiency of the reference amplification are approximately equal. A sensitive method for assessing if two amplicons have the same efficiency is to look at how the cycle number difference between ICK1 and \( \beta \)-actin (\( \Delta C_T \)) varies with template dilutions. The absolute value of the slope of log RNA versus \( \Delta C_T \) is smaller than 0.1, the relative efficiency should be considered as equal according to (Relative Quantification of Gene Expression, User Bulletin #2, Applied Biosystems). Figure 4.6 gave a slope of log RNA versus \( \Delta C_T \) equal to -0.08, which pass this examination.

### 4.3.1.2.2 Determination of ICK1 transcriptomes in transgenic \textit{N. Tabacum}

The total RNA was isolated from the leaf tissue of 15 transgenic \textit{N. Tabacum} plants with or without 40-hour 20mg/L \( \beta \)-estrodiol induction. The RNA from cell line 12-9 and wild type \textit{N. tabacum} was used to synthesized cDNA and serve as “no template control” (NTC).
According to the optimized condition, 1ug total RNA was used to synthesize cDNA by 100 units SuperScript™ III reverse transcriptase in a 20 µl reaction volume. 2 µl RT-reaction mixture (100 ng total RNA) was loaded onto each real-time PCR reaction based on the linear dynamic range of both ICK1 cDNA and N. Tabacum β-actin. After NTC was set up as 1 based on “Delta-delta model” presented by PE Applied Biosystems (Perkin Elmer, Forster City, CA), the transcripts level of explants were normalized to NTC as shown in figure 4.7. All of the tested cell lines transcribed the target gene (ICK1) at slightly varying levels (approximately from 100 to 300) without induction, which could be due to the constant level of phytoestrogen metabolized by N. tabacum.

The XVE promoter in all the tested cell lines responded effectively to the 20mg/L β-estrodiol after 40 hours. Six independent lines (#4, #18, #19, #21, #24, #28) had over ten-fold induction. The transcripts level in the induced samples reached as high as 39554 in cell line #4. Cell line #21 showed lowest leaky expression level and most sensitive induction (over 100 fold induction). Three independent cell lines (#3, #16, #21) that showed relatively lower leaky expression were chosen for further characterization.

4.3.2 Cell cycle synchrony of N. tabacum suspension culture by expressing ICK1

4.3.2.1 The growth behavior, cell morphology and physiology of ICK1 transgenic cell lines

The culture growth of cell line #16 and #21 was studied in 500 ml rotary flask over 12 days. 20 mg/L 17-β-estrodiol was added to both ICK1-expressing cultures (cell line #16, #21) and proliferation-competent cultures (cell line 12-9) at the beginning of the cultivation. No specific growth inhibition and cell morphology change were noted in the
proliferation-competent culture 12-9 (figure 4.8 and figure 4.10), indicating that 20 mg/L β-estradiol did not cause any significant adverse effect in the tobacco suspension cells. After 6-day induction suspension cells were stained by fluorescein diacetate (FDA) and observed under microscope. Morphologically, suspension cells from ICK1-expressing double transgenic lines were longer and leaner compared to the ones from the background lines (figure 4.10), and the percentage of viable elongated cells increased with the addition of inducer (less than 10% in the background line, about 30% in the non-induced samples, and about 45% in the induced samples). The cell length ranges about 3 to 5 times longer than proliferation-competent cells. Such a morphology change was also reported in cell-cycle arrested BY-2 cells by expressing CDKa:1.N146 negative mutant (Joubes et al. 2004). The elongation was putatively regarded as the consequence of cellular growth when cell division was blocked (Joubes et al. 2004). As a characteristic of the suspension culture, the prolonged cells seem more easily to tangle together and form more aggregates in transgenic ICK1 expressing \textit{N. tabacum} cultures. As presented in figure 4.8, on the basis of cell dry weight, cell growth in the non-induced #16 and #21 cultures is comparable to that in the control culture; while in the β-estradiol induced cultures, considerable reduction in cell growth was noted. After 10-12 days of cultivation, the maximum dry cell weight in the control culture reached 7.4 g/l, while only about half of that level was reached in the induced #16 and #21 cultures. The induced ICK1 expression led to about 50% reduction in specific growth rate. When the growth was evaluated based on viable cell numbers, even the non-induced #16 and #21 cultures displayed a slower growth rate than the control culture. The slightly slower growth in the non-induced #16 and #21 cultures was likely due to the leaky expression of ICK1. The
induced #16 and #21 cultures still showed slight growth in dry biomass (approximately 1.5 fold of initial dry biomass) that was probably contributed by those cells escape from the cell-cycle arrest and the biomass growth in cellular elongation. Although cell line #21 possessed 3 times higher transcripts of ICK1 than cell line #16, no obvious differences in growth behavior were found between those two cell lines in the presence of β-estrodial, suggesting that cell cycle arrest in *N. tabacum* suspension cultures could occur at a relatively low level of ICK1, beyond that low threshold the cell cycle might have approached almost complete arrest.

For the cytostatic gene p27-mediated proliferation-control in CHO suspension cultures, no difference was observed in specific glucose uptake rates compared to proliferation-competent cultures (Mazur et al. 1999). Here, we studied the metabolic activity in terms of substrate uptake of the ICK1-expressing *N. tabacum* suspension culture and presented the glucose consumption in figure 4.9 on a time-course basis. It was assumed that the glucose consumption was exclusively due to the metabolic activity of viable cells, and the maximum specific glucose uptake rates were 1.195 (g/g dry biomass of viable cells/day) and 1.424 (g/g dry biomass of viable cells/day) for cell line #16 and cell line #21 in the absence of 17-β-estrodial, while the glucose uptake rate was approximately 3 times lower in both of cell lines #16 and #21 in the presence of 17-β-estrodial. Unlike the mammalian system, it demonstrated that ICK1-based specific G1 phase arrest decelerate the metabolic activity in plant cells.

The culture viability (viable cells distinguished by FDA staining) was also monitored on a time-course basis and presented in figure 4.11. Unfortunately, the culture viability of #16 and #21 seemed to be influenced negatively by the expression of ICK1.
The viability of freshly inoculated control culture initially showed an increase for about 10% and remained at around 80% for about ten days of cultivation. During that same period, the viability of both non-induced and induced ICK1-expressing double transgenic cell suspension cultures fluctuated around 50%. All cultures showed considerable drop in viability from day 9 to day 12. G1-phase cell cycle arrest was extensively reported to induce programmed cell death (apoptosis) in eukaryotic cells. p53 as an essential tumor suppressors in mammalian cells induces apoptosis by activating apoptosis-inducing gene, p53AIP or cyclin dependent kinase inhibitor p21waf to remove cells with serious DNA damage or stress (Kadota et al. 2004; Lane 1992; Oda et al. 2000; Oren 1994); In BY2 cells, an proteinaceous elicitor cryptogein induced G1 phase cell cycle arrest was also found to precede apoptosis (Kadota et al. 2004). To further distinguish whether the low culture viability was due to unspecific necrotic cell death or apoptosis, the typical biochemical hallmark of apoptotic cells, genomic DNA fragmentation, was detected by terminal deoxynucleotidyl transferase (tdt)-mediated dUTP nick end labeling (TUNEL) (Gavrieli et al. 1992). As shown in figure 4.12, the positive control, cultures overnight-treated by 100 mM, H2O2 showed a high percentage (about 95%) positive TUNEL staining (TMR red fluorescence). Although cell line #21 without induction and 12-9 culture showed a low proportion of positive labeled cells (less than 3-4%), no statistically significant positive results (less than 1-2%) were found after ICK1 expression. Similar results were also observed in the cell death of A. thaliana trichome cells expressing ICK1/KRP1 (Schnittger et al. 2003). The expression of CDK inhibitors-ICK in A. thaliana leaves and dominant-negative CDKa mutant in N. tabacum BY2 cells both resulted in less cell number and an expanded cell size (Joubes et al. 2004; Wang et al.
2000). The ICK1 double transgenic N. tabacum suspension cells here are also much more elongated (and also with higher cellular dry weight) compared with the control cells, and the former also have lower DNA content (mostly 2C; figure 4.14), and hence these double transgenics have a lower nucleocytoplasmic ratio (DNA content per cell size) as well. It has been suggested that very low nucleocytoplasmic ratio may lead to necrotic cell death (Schnittger et al. 2003) because there is insufficient amount of DNA and perhaps other biomolecules to support the metabolism and energy balance of the enlarged cells. It is unclear whether there exists a threshold nucleocytoplasmic ratio in tobacco cells, below which necrotic cell death occurs. Moreover, the enlarged cell size we observed in the ICK1 double transgenics may come from enlarged vacuole, rather than the cytoplasm.

4.3.2.2 The arrest of G1/S transition in ICK1-expressing N. tabacum suspension cells

The arrest of G1/S transition was evaluated by analyzing the percentage of certain cell populations that distributed in various cell cycle phases. The nucleus isolated from 6-day old induced #16 and non-induced #16 suspension cells were treated by propidium iodide and resolved in flow cytometry analysis. Doublets and higher nuclei aggregates were removed by screening the singlet populations in pulse shape analysis. Cell line 12-9 served as a background control. The obtained flow cytometry histograms were fitted to Watson Pragmatic Model, the "Dean-Jett-Fox" model, and the "2-populations" model respectively (Fox 1980; Watson 1992; Watson et al. 1987), in which the Watson algorithm gave the best fitting results in terms of smaller roots of mean squared errors. As demonstrated in figure 4.13, the flow cytometry analysis revealed that the leaky
expression of ICK1 in absence of 17-β-estrodiol reduced 18.5% of cells in G2 phase and expression of ICK1 in presence of 17-β-estrodiol lead to 19.4% drop in G2-phase population and 10% decrease in S-phase population in transgenic *N. tabacum* suspension cultures. Concomitantly, G1-phase population accumulated to 91.9%, indicating that ICK1-mediated G1 phase arrest is highly effective in transgenic *N. tabacum* suspension cultures.

The recombinant ICK1 was effective in inhibiting the histone H1 kinase activity of p13<sub>suc1</sub>-associated kinases from cultured cells of heterogous *Brassica napus* (Wang et al. 1998), *Arabidopsis thaliana* seedlings, leaves and floral tissues (Wang et al. 1998; Zhou et al. 2003). By an *in vitro* functional kinase activity inhibition assay with CDKa complex pulled down by p13<sub>suc1</sub>-agarase beads from extracts of the ICK1-transgenic lines, the CDKa-like kinase activity by the level of phosphorylation of histone H1 (estimated by Western blotting with a anti-phosphorilated-H1-antibody) was determined (figure 4.14). The level of cdc2a complied with a good correlation with p13<sub>suc1</sub>-associated Kinase activity in most tissues except stem(Wang et al. 1998). After normalized the phosphorylated Histone H1 according to the purified cdc2a level, it indicates a significant decrease of phosphorylated Histone H1 in the induced ICK1 lines compared to the wild type control sample (over 80% decrease) and non-induced sample Figure 4.14 C. It clarified that the His-tagged ICK1 protein was expressed properly and retains its inhibitory characteristics. Hence consistent results between flow cytometry analysis and CDKa kinase activity assay were achieved to enforce the statement of specific G1/S transit point arrest in these ICK1 transgenic suspension culture.
4.3.3 The effect of G1/S arrest on reporter protein (GFP) productivity

The effect of ICK1 expression and the G1/S-phase arrest caused by it on the levels of the GFP reporter protein were estimated by Western blotting. For that purpose, both total intracellular and extracellular (secreted) protein were extracted from both the ICK1 cell lines (#16 and #21) and background control (cell line 12-9) after 6 day induction, equal amount total soluble protein (10 μg and 0.3 μg, respectively) from the extracts were separated under SDS-PAGE denaturing conditions and probed with an anti-GFP antibody.

The colometrical band intensity of the western blot was taken as the mass of GFP in all the extracts for relative comparison of GFP productivity (overall GFP content including both secreted GFP and intracellular residual GFP) within different cell lines. The recombinant GFP was assumed exclusively produced by viable cells (distinguished by FDA staining), thus the specific production of recombinant GFP was normalized based on number of viable cells or dry biomass of viable cells (as shown in figure 4.14A and B). Unfortunately, a decrease of GFP production closely correlated with the expression of ICK1 and the culture growth arrest. As shown in figure 4.16 A and B, about 3-fold decrease in specific productivity was observed after expression of ICK1. This inverse-correlation between model protein (GFP) and cytostatic protein (ICK1) probably acts initially on the mRNA levels determined by quantitative RT-PCR against both GFP transcripts and ICK1 transcripts (data not shown). However, the reason causing the significant decrease in the GFP expression in the uninduced XVE-ICK1 cells relative to the background control remains questionary and unexpected, which was suspected as results of the leaky expressing of ICK1. Furthermore, the total protein synthesis (based on
total protein per cell or cell dry weight) was noted being negatively affected by the ICK1 expression as well (data not shown). This observation is understood as a consequence of lowered cellular metabolic activity (indicated by the lower substrate consumption rate) that negatively affected the protein synthesis machinery.

The ICK1 counterpart p27^{Kipl} from the mammalian CDK inhibitor proteins (CIP/KIP) family had been conditionally over-expressed in CHO cells without deleterious consequences and it actually increased by 10- to 15-fold the specific protein productivity compared to proliferation-competent control cell lines (Mazur et al. 1998). However, the observations that the expression of AtlCK1 leads to unexpected decrease of viability and lower levels of recombinant protein production in the tobacco cell suspension culture made us re-assess the possible effects of the ICK1-caused G1/S-phase arrest on the physiology of \emph{N. tabacum} cells. Several differences between the mammalian and plant systems are noted below.

First, in animals the CIP/KIP proteins are inhibitors of the E- and A-cyclin-dependent CDK complexes, but they are also required as assembly proteins for the formation of cyclinD-dependent complexes with Cdc4 and Cdc6 and those triple CyclinD-CDK-KIP complexes are catalytically active towards the Retinoblastoma protein (pRb, LaBaer et al., 1997). Due to the sequestration of p27^{Kipl} in the CyclinD-dependent Cdc4 and Cdc6 complexes, CyclinE-dependent Cdc2 complexes, which complete the phosphorylation of pRb are active in a concentration-dependent manner - at low amounts of p27^{Kipl} they are catalytically active towards pRb, but an excess of p27^{Kipl} inactivates them. Thus, under certain conditions p27^{Kipl} might also exercise a positive function for the entry into the S-phase (Sherr and Roberts, 1999). In plants, so far no
positive function for the cell-cycle progression or the cell physiology in general has been assigned to ICK1. Additionally in plants until now only one type of CyclinD-dependent kinase (Cdc2a/CDKA) has been described to be implicated in the pRb phosphorylation at the G1/S transition (Boniotti and Gutierrez 2001, Nakagami et al 2002) therefore there might be differences in the way of regulation of the G1/S transition between plant and animal systems. Structurally, the p27Kip1 and ICK1 share only a limited homology in the kinase-interaction domain, localized at the N-terminus of p27Kip1 and at the C-terminus of ICK1 (Wang et al., 1997); therefore differences in their regulation, functions and effects might be expected as well.

In animals, in addition to its role as a CDKI, p27Kip1 is also a putative tumor suppressor, a factor for cell differentiation and is also suspected to be involved in promoting apoptosis (Lloyd et al, 1999; Philipp-Staheli et al., 2001). Similarly, in plants ICK1 miss-expression in A. thaliana trichomes was reported to initiate cell death (Schnittger et al., 2003) and also compromised the general cell viability of our system (N. tabacum cv. Xanthi cell suspensions). Unfortunately, until now the type of cell death caused by the presence of ICK1 in the cells is not entirely clear. In our study, we could not detect significant signs of programmed cell death neither by TUNEL assay nor by DNA-laddering (fragmentation) separation. Schnittger et al. (2003) reported some apoptotic-like symptoms in ICK1-expressing trichomes e.g. disappearance of the chromocenters and the nucleoli; however, that phenomenon of nucleoli disintegration can also be a sign of reduced ribosome biogenesis due to the G1/S-phase cell cycle arrest and the low nuclear DNA content caused by it (Sugimoto-Shirasu and Roberts, 2003). The result of that insufficiency of nuclear DNA can cause a classical case of necrosis e.g. due
to the inability to sustain the vital functions of the enlarged cells (protein synthesis, metabolic and energy functions, etc) at the necessary levels.

The concept of a "nucleo-cytoplasmatic ratio" (i.e. that the cell has some control mechanisms that ensure that the DNA amount of the cell is sufficient to support the optimal functioning of the cytoplasm) has been widely discussed and considerable evidence to support it has been gathered from animal studies, but to our knowledge there has not been quantitative reports for plant systems. Nevertheless, it has been well characterized via classical genetic methods that tetra- and polyploid plants are usually bigger than their diploid relatives, and that high nuclear DNA content (which initially is a result of endoreduplication cycles) causes enlargement of the plant cells. A classical example for the later correlation is found in the polyploid periclinal chimeras of *Datura stramonium* meristems, in which each discrete cell layer has a different ploidy with the corresponding nuclear and cell-size changes (Satina and Blakeslee, 1941). Polyploidy with its multiplication of genes might have a positive effect on a variety of cell processes e.g. ribosome formation, global gene expression levels, etc.; therefore, since ICK1 expression arrests the plant cells before the DNA-replication phase and in general leaves the cells with low DNA content (estimated by flow cytometry of nuclei isolated from non-induced and induced transgenic lines and from background line samples), it is possible that the decreased viability we observed in ICK1-transgenic cell lines is a result of insufficient DNA content for sustaining the functions of the elongated cells.

In addition, plant cells in contrast to animal cells are elongating via expansion of their vacuole, after loosening the cell walls and deposition of new structural units there; animal cells however grow in size mostly by increasing their cytoplasmatic biomass.
Furthermore, for a given amount of DNA and its corresponding optimal mass of cytoplasm a plant cell can achieve various cell volumes through differential vacuolization and cell expansion (Sugimoto-Shirasu and Roberts, 2003) under the influence of the complex interactions between internal and environmental factors. The consequence is that the protein synthesis potential of a plant cell is in direct correlation not with the general volume of the cell, but with the volume and activity of its DNA and cytoplasmatic fractions. One of all those "mass-to-volume" correlations or a combination of them might have also contributed significantly for the observed decrease of recombinant GFP production in the G1/S-arrested suspension cultures.

The still unresolved issue about a possible S-phase cell-stage specificity of the activity of the 35S promoter used to drive the expression of GFP in our system brings out an additional factor to reckon with when assessing the possible causes of the recombinant GFP protein production decrease. Nagata et al. (1987) reported oscillations of the activity of the 35S promoter, measured as changes in the activity of the chloramphenicol transferase (CAT). The maximum peak of 35S-driven CAT activity coincided with the minimal mitotic index of the cells i.e. during the S phase and was minimal between the M and the G1-phase; in contrast the nopaline synthase (nos) promoter used also in the same study for the expression of CAT showed no such cell-cycle dependence. It is possible then that in the pre S-phase arrested cells the 35S promoter is not active to its full potential and that leads to a decrease of the protein controlled by it.

As for the observed lower viability of the ICK1-transgenic cell suspensions, another possibility is that the constitutive expression of the hybrid XVE transcriptional factor that controls the ICK1 expression might cause some physiological aberrations in N.
*tabacum* cells. The XVE system has been characterized mostly in *Arabidopsis* and there it showed no drawbacks, but to our experience with *N. tabacum* plants and also in *N. benthamiana* plants (Dr. M Boulton, JIC, personal communication) the XVE system besides being leaky in *Nicotiana* species seems to cause some developmental defects - shorter stature and internodes, thick and short roots, reduced apical dominance, etc (data not shown). These growth and developmental defects could not be attributed specifically to the XVE fusion protein but certainly have their origin in some changes of the physiology and development of certain actively-dividing types of cells in the plant e.g. meristematic clusters; therefore the possible role of the hybrid fusion XVE transcription factor for the changes of the physiology and viability of tobacco cells was not fully characterized in the present study and deserves more detailed further investigation.

**4.4 CONCLUSION**

The controllable growth regulation of double transgenic *N. tabacum* suspension system was established by genetically expressing *Arabidopsis thaliana* cycline dependent kinase inhibitor (ICK1) and green fluorescence protein (GFP). The adding of 17-β-estrodiol switch on the expression of ICK1 in these double transgenic suspension cultures. It lead the culture to an inhibited growth phase from competent proliferation phase. The flow cytometry analysis of these ICK1-expressing cultures indicates a high efficient cell-cycle synchrony (over 90% G1-phase population), which brought an effective method in further cell cycle study of plant suspensions. The original tentative concepts to prolong the culture life and increase the recombinant protein productivity seems to be infeasible due to the low culture viability and impaired protein synthesis machinery.
REFERENCES


Table 4.1 Conditions for quantitative RT-PCR.

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<td>Calculated threshold using the <strong>maximum curvature approach</strong>.</td>
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<td>Per-well baseline cycles have been determined automatically.</td>
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Figure 4.1 The plasmid map of binary vector pXVE carrying 6His-ICK1.
Figure 4.2 PCR amplification of ICK1 against genomic DNA of selected transformants.

+control: The pXVE binary vector carrying ICK1.
-control: Genomic DNA from W.T. N. Tabacum and 10-6
Figure 4.3. Gel-based RT-PCR of selected transgenic *N. Tabacum*. "+" represents for plants were induced by 20mg/L β-estradiol. "-" represents for plants without induction. The positive control was pXVE-6His-ICK1 as template.
Figure 4.4 A. real-time PCR amplification curve.

B. Melting curve.
Figure 4.5 Linear dynamic range of ICK1 and β-actin cDNA.
Figure 4.6 Relative Amplification Efficiency of ICK1 and internal control β-actin.
Figure 4.7 ICK1 transcripts determination by quantitative RT-PCR.

- **Induced cell lines**
- **Non-Induced cell lines**
Figure 4.8 Transgenic ICK1 culture growth curve.
A) Dry cell weight v.s. Culture time
B) Packed cell volume v.s. Culture time
Figure 4.9 Time course experiments of glucose consumption.
Figure 4.10. Morphology of the transgenic ICK1-expressing cells. Cells from lines 12-9, #21 and #16 were examined under phase-contrast microscope 6 days after induction (column A and C). The cells in column A and C were highlighted by fluorescein diacetate (FDA) viability staining (column B and D respectively). The white bar represents 100 micrometers.
Figure 4.11 Time-course study of ICK1 transgenic culture viability. The general culture viability was determined by number of fluorescein diacetate (FDA) stained cells divided by total cell number.
Figure 4.12 In situ detection of apoptotic hallmark – DNA cleavage in transgenic ICK1 expressing N. tabacum suspension cells. The suspension cells were tested after 10 days cultivation. (+) represents suspension cells cultivated in presence of 20mg/L 17-β-estrodio 1. (-) represents suspension cells cultivated in absence of 20mg/L 17-β-estrodio 1. Positive control: The suspension cells of cell line 12-9 were treated by 100mM H₂O₂ for 12 hours.
Figure 4.13 Flow cytometry histogram and cell cycle distribution of nuclei from ICK1 transgenic suspension culture stained with propidium-iodide. A. 12-9 as background control. B. cell line #16 cells were cultivated in absence of inducer for 6 days. C. cell line #16 culture were cultivated in the presence of 17-ß-estradiol for 6 days.
Figure 4.14 The inhibitory activity of recombinant AtlCK1 against p13^{suc1}-agarose purified CDKa kinase. A) Western blot detection of phosphorylated histone H1. B) purified CDKa amounts detected by western blot. C) The CDKA kinase activity represented as band intensity of detected phosphorylated histone H1 divided by the band intensity of detected purified CDKA. "+" represents in the presence of 17-β-estradiol. "-" represents in the presence of 17-β-estradiol.
Figure 4.15 Western blots with an anti-GFP antibody against 10 µg of intracellular (A) and 0.3 µg of extracellular (B) protein extracts from non-induced (-) and induced (+) ICK1-expressing *N. tabacum* lines #16 and #21
Figure 4.16. Normalized GFP production in ICK1 cultures. A. GFP production normalized based on dry biomass of viable culture cells. B. GFP production normalized based on culture viable cell numbers.
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APPENDIX A – Derivation of the geometric fractional factor $f'(x, y, R)$ defined in equation (2)

As depicted in Figure 3.3B, $c$ is the center of the probe tip surface $T$ (which is parallel to the $y$-$z$ plane) with a radius of $R$, $O$ represents the point light (fluorescence) source. The horizontal and the vertical distances between points $O$ and $c$ are $x$ and $y$, respectively. Arcs $f'g'$ and $m'n'$ are respectively the projection of diameters $fg$ and $mn$ of the probe tip cross-section $T$ onto a virtual spherical surface $S'$ (not shown) with a center at point $O$ and an arbitrarily chosen radius $p$. Our objective is to calculate the value of $f'(x, y, R)$ which represents the fraction of light (fluorescence emitted from point $O$) that reaches the probe tip surface $T$. This is equivalent to the proportion of the area $S^*$ (Fig. 3B) on the entire virtual spherical surface $S'$ ($4\pi p^2$). Suppose that $p$ is an arbitrary point on the probe tip surface with polar coordinates $(r, \theta)$, then, the spatial vector from point $O$ to point $p$ and its length are respectively expressed by

$$\overline{Op} = x\hat{i} + (y + r \cos \theta)\hat{j} + r \sin \theta \hat{k}$$

$$\overline{Op} = \sqrt{x^2 + (y + r \cos \theta)^2 + (r \sin \theta)^2}$$

$$= \sqrt{x^2 + y^2 + r^2 + 2yr \cos \theta}$$

(A1)

(A2)

where $\hat{i}$, $\hat{j}$ and $\hat{k}$ are the unit vectors along the rectangular axes $x$, $y$ and $z$ respectively. Furthermore, let the projection of point $p$ from point $O$ onto surface $S^*$ be $p'$, then

$$\overline{Op'} = \frac{P_{op}}{P_{op}} \overline{Op} = \zeta_1(r, \theta)\hat{i} + \zeta_2(r, \theta)\hat{j} + \zeta_3(r, \theta)\hat{k}$$

(A3)

where

$$\zeta_1(r, \theta) = x \frac{P_{op}}{P_{op}}, \quad \zeta_2(r, \theta) = (y + r \cos \theta) \frac{P_{op}}{P_{op}}, \quad \zeta_3(r, \theta) = r \sin \theta \frac{P_{op}}{P_{op}}$$

(A4)

The normal vector at point $p'$ on the spherical surface $S^*$ is given by

$$\overline{N} = \begin{vmatrix} \hat{i} & \hat{j} & \hat{k} \\ \frac{\partial \zeta_1}{\partial r} & \frac{\partial \zeta_2}{\partial r} & \frac{\partial \zeta_3}{\partial r} \\ \frac{\partial \zeta_1}{\partial \theta} & \frac{\partial \zeta_2}{\partial \theta} & \frac{\partial \zeta_3}{\partial \theta} \end{vmatrix} = N_1\hat{i} + N_2\hat{j} + N_3\hat{k}$$

(A5)

where
We note that

\[
\begin{align*}
\frac{\partial \zeta_1}{\partial r} &= -x (r + y \cos \theta) \rho / (\overline{O p})^3 \\
\frac{\partial \zeta_1}{\partial \theta} &= x y r \sin \theta \rho / (\overline{O p})^3 \\
\frac{\partial \zeta_2}{\partial r} &= (x^2 \cos \theta - y r \sin^2 \theta) \rho / (\overline{O p})^3 \\
\frac{\partial \zeta_2}{\partial \theta} &= -r (x^2 + r^2 + y r \cos \theta) \sin \theta \rho / (\overline{O p})^3 \\
\frac{\partial \zeta_3}{\partial r} &= (x^2 + y^2 + y r \cos \theta) \sin \theta \rho / (\overline{O p})^3 \\
\frac{\partial \zeta_3}{\partial \theta} &= r [(x^2 + y^2 + r^2) \cos \theta + y r (1 + \cos^2 \theta)] \rho / (\overline{O p})^3
\end{align*}
\] (A6)

\[
\begin{align*}
N_1 &= \frac{\partial \zeta_2}{\partial \theta} \frac{\partial \zeta_3}{\partial r} - \frac{\partial \zeta_3}{\partial \theta} \frac{\partial \zeta_2}{\partial r} = x^2 r \rho^2 / (\overline{O p})^4 \\
N_2 &= \frac{\partial \zeta_3}{\partial \theta} \frac{\partial \zeta_1}{\partial r} - \frac{\partial \zeta_1}{\partial \theta} \frac{\partial \zeta_3}{\partial r} = (y + r \cos \theta) x r \rho^2 / (\overline{O p})^4 \\
N_3 &= \frac{\partial \zeta_1}{\partial \theta} \frac{\partial \zeta_2}{\partial r} - \frac{\partial \zeta_2}{\partial \theta} \frac{\partial \zeta_1}{\partial r} = (r \sin \theta) x r \rho^2 / (\overline{O p})^4
\end{align*}
\] (A7)

We note that

\[
\frac{N_1}{x} = \frac{N_2}{y + r \cos \theta} = \frac{N_3}{r \sin \theta} = x r \rho^2 / (\overline{O p})^4
\] (A8)

This verifies that the normal vector \( \overline{N} \) on the spherical surface \( S^* \) at point \( p' \) indeed coincides with the vector \( \overline{O p} \).

\[
|\overline{N}| = \sqrt{N_1^2 + N_2^2 + N_3^2} = \left[ x r \rho^2 / (\overline{O p})^4 \right] \sqrt{x^2 + (y + r \cos \theta)^2 + (r \sin \theta)^2} = x r \rho^2 / (\overline{O p})^3
\] (A9)

By considering symmetry of light distribution about the plane passing through points \( O, c \) and \( e \), \( S^* \) area \( \left( A_s \right) \) is determined as (Kreyszig 1999)

\[
A_s = \int_S dA = \int_t |\overline{N}| \rho \theta = 2 \rho^2 \int_t \rho \sin \theta |\overline{N}| \rho \theta d\theta = 2 \rho^2 \int_t \rho \sin \theta |\overline{N}| \rho \theta d\theta
\] (A10)

and hence \( f' \) is calculated as

\[
f'(x, y, R) = \frac{A_s}{4 \pi \rho^2} = \frac{1}{2 \pi} \int \rho \int \frac{x r}{\left( \sqrt{x^2 + y^2 + r^2 + 2 y r \cos \theta} \right)} \rho \theta d\theta
\] (A11)

In the case of \( y = 0 \) the above equation reduces to
\[ f''(x, R) = \frac{1}{2\pi} \left[ \frac{x}{(\sqrt{x^2 + r^2})^3} \right]_R^\theta \] \[ = \frac{1}{2} \left( 1 - \frac{x}{\sqrt{x^2 + R^2}} \right) \] (A12)

which is the result given by Wang and Simmons (1991).

**APPENDIX B – The Extended Kalman Filters**

Let \( \xi = [C_x, C_s, C_p]^T \) as the state vector, Eqs. (14) and (17) or (18) may be respectively expressed as

\[
\dot{\xi} = \phi(\xi) + w; \quad \xi|_{t=0} = \xi_0
\]
\[
y = h(\xi) + v
\]
where \( \phi(\cdot) \) is the dynamic state function and \( h(\cdot) \) is the measurement function, both system noise \( w(=[W_x, W_s, W_p]^T) \) due to modeling error and unknown disturbances and measurement noise \( v(=V_F) \) are assumed to be independent zero-mean white noises.

For continuous measurements, the following EKF algorithm gives the optimal state estimate \( \hat{\xi}(t) \) based on the available measurements up to current time \( t \):

\[
\dot{\xi} = \phi(\hat{\xi}) + PC^T R^{-1}[y - h(\xi)]; \quad \xi|_{t=0} = \xi_0
\]
where \( P \) is governed by Eq. (20), in which

\[
A = \left[ \begin{array}{ccc}
L_a & L_b \hat{C}_x & 0 \\
-\left\{ Y_s \hat{L}_a \hat{b}_s + \hat{\mu} \hat{v}_b \hat{C}_x \right\} \frac{1}{\hat{b}_{sX}} & -\left\{ L_b Y_s - \hat{\mu} \hat{v}_b \right\} \frac{\hat{C}_s}{\hat{b}_{sX}} & 0 \\
k_o \hat{L}_a + k_N & k_o L_b \hat{C}_x & -k_D \\
\end{array} \right]
\]
(A16)

where

\[
\hat{b}_{sX} = 1 - \hat{v}_b \hat{C}_s, \quad Y_s = \frac{1}{Y_{X|S}} - \hat{v}_b \hat{C}_s
\]
(A17)

When the Contois model (Eq. 15) is used,

\[
\hat{\mu} = \mu_{\text{max}} \frac{\hat{C}_x}{BC_x + \hat{C}_s}, \quad L_a = \frac{\partial (\hat{\mu} \hat{C}_x)}{\partial \hat{C}_s} = \mu_{\text{max}} \left( \frac{\hat{C}_s}{BC_x + \hat{C}_s} \right)^2, \quad L_b = \frac{\partial \hat{\mu}}{\partial \hat{C}_s} = \frac{\mu_{\text{max}} B \hat{C}_s}{(BC_x + \hat{C}_s)^2}
\]
(A18)

When the Monod model (Eq. 16) is used,
\[
\hat{\mu} = \mu_{\text{max}} \frac{\dot{C}_s}{K_s + \dot{C}_s}, \quad L_a = \frac{\partial(\hat{\mu} - \dot{C}_s)}{\partial \dot{C}_s} = \hat{\mu}, \quad L_b = -\frac{\partial \hat{\mu}}{\partial \dot{C}_s} = \frac{\mu_{\text{max}} K_s}{(K_s + \dot{C}_s)^2}
\]

If only \( F \) is chosen as the measured variable, then

\[
C = c_1 = \begin{bmatrix} f_0 \gamma - 2b_0 \dot{C}_s \tau S[(1 + \delta L)e^{-\delta L} - 1] / \delta^2 & 0 & b_0 \gamma \end{bmatrix}
\]

with

\[
\gamma = (1 - e^{-\delta L}) / \delta, \quad \delta = S + 2\tau C_s \]

For EKF with intermittent measurements, two approaches are examined: a two-stage prediction/correction approach, and a zero-order-holder (ZOH) approach. For the two-stage prediction/correction approach, a continuous prediction of the states \( \xi(t) \) and the estimation error covariance \( P(t) \), during the “prediction” stage of the time interval \( t \in (t_{k-1}, t_k) \) before the time instant \( t_k \), is made based on the state model and previous estimation results, \( \hat{\xi}(t_{k-1}) \) and \( P(t_{k-1}) \), i.e.

\[
\begin{align*}
\hat{\xi}(t | t_{k-1}) &= \Phi [\hat{\xi}(t | t_{k-1})], \\
\hat{\xi}(t | t_{k-1})|_{t=t_k} &= \hat{\xi}(t_{k-1}) \\
\hat{P}(t | t_{k-1}) &= \Phi(t | t_{k-1}) A_{k-1}^T + A_{k-1} \hat{P}(t | t_{k-1}) + Q, \quad \hat{P}(t | t_{k-1})|_{t=t_k} = P(t_{k-1})
\end{align*}
\]

where

\[
A_{k-1} = \frac{\partial \Phi}{\partial \xi}|_{\xi = \hat{\xi}(t_{k-1})}
\]

Subsequently in the “correction” stage of the \( k \)-th time interval \( t \in (t_{k-1}, t_k) \) (i.e. at \( t=t_k \)), a correction is made on \( \hat{\xi}(t_k | t_{k-1}) \) and \( P(t_k | t_{k-1}) \) by means of the EKF and the newly available measurement \( y(t_k) \), i.e.

\[
\begin{align*}
\hat{\xi}(t_k) &= \hat{\xi}(t_k | t_{k-1}) + K(t_k) \{ y(t_k) - h[\hat{\xi}(t_k | t_{k-1})] \} \\
P(t_k) &= P(t_k | t_{k-1}) - K(t_k) C_{k|k-1} P(t_k | t_{k-1}) \\
K(t_k) &= P(t_k | t_{k-1}) C_{k|k-1}^T [C_{k|k-1} P(t_k | t_{k-1}) C_{k|k-1}^T + R]^{-1}
\end{align*}
\]

where

\[
C_{k|k-1} = \frac{\partial h}{\partial \xi}|_{\xi = \hat{\xi}(t_{k-1})}
\]

A second approach involves the zero-order-holder (ZOH) EKF. For each sampling interval \( t \in [t_{k-1}, t_k) \) the previous measurement \( y(t_{k-1}) \) is kept up to the new sampling time instant \( t_k \), i.e.

\[
y(t) = y(t_{k-1}), \quad t \in [t_{k-1}, t_k)
\]

while the on-line filtering algorithm takes the same form as that of the continuous-time filtering:
\[
\begin{align*}
\dot{\xi} &= \varphi(\hat{\xi}) + \tilde{P} C^T \bar{R}^{-1} [y - h(\hat{\xi})] \\
\dot{\tilde{P}} &= \tilde{P} A^T + A \tilde{P} - \tilde{P} C^T \bar{R}^{-1} C \tilde{P} + Q
\end{align*}
\]

(A29)

where \( \tilde{P} \) is the covariance matrix of state estimation error in the ZOH EKF, and

\[
A = \varphi_x(\hat{\xi}(t)) = \frac{\partial \varphi}{\partial \xi} \bigg|_{\xi = \hat{\xi}(t)} \quad \quad C = h_x(\hat{\xi}(t)) = \frac{\partial h}{\partial \xi} \bigg|_{\xi = \hat{\xi}(t)}
\]

(A30)

To obtain a satisfactory filtering result using the ZOH EKF, the \( \bar{R} \) value can be estimated as follows. The error introduced by the ZOH approximation on the measurement signals, \( V_{ZOH} \), may be viewed as independent of original measurement noise (which has a variance of \( R \)). If the sampling period \( \Delta \) is not too large and the average slope of the measured \( F \) curve within the \( i \)th sampling interval \([ (i-1) \Delta, i \Delta ) \) is \( \lambda_i \), then \( V_{ZOH} \) can be considered as to vary linearly from 0 to \( \lambda_i \Delta \). Thus,

\[
\bar{R} = R + Var V_{ZOH} \approx R + \frac{1}{N_s} \sum_{i=1}^{N_s} \left[ \int_{(i-1)\Delta}^{i\Delta} \lambda_i [t - (i - 1) \Delta]^2 \, dt \right]
\]

\[
= R + \frac{1}{N_s} \sum_{i=1}^{N_s} \left[ \frac{1}{3} (\lambda_i \Delta)^2 \right] = R + \frac{1}{3} (\lambda \Delta)^2 = k_r (\lambda \Delta)^2
\]

(A31)

where \( \lambda \) is the mean squared root slope of the measured \( F \) curve within \( N_s \) sampling periods, and \( k_r \) is an empirical coefficient that takes into account the effect of the original measurement noise \( R \). From Eq. A31, \( k_r \) should be greater than 1/3. In our case the mean squared root slope \( \lambda \) is approximately \( 2.2 \cdot 10^{-5} \, \text{d}^{-1} \), and the sampling period \( \Delta \) is set at 0.5 d. From simulations we noted that estimation was not significantly affected when \( k_r \) was varied from 0.4 to 1. By setting \( k_r = 0.4, \bar{R} \approx k_r (\lambda \Delta)^2 = 0.4 \cdot (2.2 \cdot 10^{-5} \cdot 0.5)^2 \approx 0.48 \cdot 10^{-10} \).